Rita Alexandra Pereira Gameiro

Licenciada em Ciências da Engenharia Química e Bioquímica

Valorization of olive pomace with subcritical water

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FACULDADE DE CIÊNCIAS E TECNOLOGIA
UNIVERSIDADE NOVA DE LISBOA

Setembro de 2016

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Acknowledgment

A realização desta dissertação de mestrado, é o fim de mais uma etapa na minha formação académica que com importantes apoios e incentivos sem os quais não se teria tornado uma realidade e aos quais estarei eternamente grata e seguramente esta secção é pequena para agradecer, como devia, a todas a pessoas que ao longo deste percurso me ajudaram e apoiaram, a cumprir os meus objetivos.

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Abstract

All over the world large quantities of agro-industrial waste are produced, which accumulate over the years, causing serious environmental problems and a devaluation of this promising biomass because it is cheap and has great potential, since it produces a wide range of products when it is processed. An example is the olive pomace (OP), a byproduct of the olive oil industry, characterized by being a very humid sludge, composed of a mixture of skin, flesh and seeds from the olives. In addition to oil, this residue contains antioxidants with potential added value for the cosmetics, food and pharmaceutical industries.

The objective of this study, is to explore the PO's potential as a source of antioxidants, by extracting these compounds with subcritical water. In parallel, the partial hydrolysis of this residue also takes place, obtaining a sugar rich liquor. For the purpose of homogenization, the PO was divided into two samples, Fine Sample (FS) and Gross Sample (GS). The GS consisted mainly of seeds residues from olive, and the particles had diameters above 1400 micrometers.

Before the extraction, both samples were chemically characterized. It was found that FS had a higher content of lipids (about 55%) and soluble sugars (about 5%). On the other hand, the GS had higher levels of insoluble sugar (about 23%) and lignin (60%). The polyphenol content obtained for both samples was less than 1%, and hydroxytyrosol was the most abundant phenolic compound.

The extraction/hydrolysis was performed only for FS in order to optimize the process, the temperatures used were 140, 170 and 200°C. The water flow and pressure were maintained constant, 10 mL/min and 60 bar, respectively. The best results were obtained at 200 °C, with a yield of soluble compounds of 30%. It was possible to extract 20.87 mg/g of phenolic compounds and a recovery of sugars of 14% wt., relative to the existing, 18.12%. It was also determined the antioxidant activity of extracts obtained in each assay, the best result was obtained for the extracts, at 200 °C, having been obtained an EC_{50} =32.83 mg/L. It also studied the stability of phenolic compounds as well as antioxidant activity. As expected, through time, the phenolic compounds will be degraded, and is more pronounced when these compounds are exposed to light and at room temperature.

Keywords: agro-industrial; residues; olive pomace; subcritical water; antioxidants; phenolic compounds

Resumo

Em todo mundo são produzidas grandes quantidades de resíduos agroindustriais, que se vão acumulando ao longo dos anos, provocando graves problemas ambientais e uma desvalorização desta biomassa promissora, pois é barata e tem um grande potencial, pois quando é processada produz uma vasta gama de produtos. Um exemplo é o bagaço da azeitona (BA), um subproduto da indústria do azeite, caracterizado por ser uma lama muito húmida, constituída por uma mistura de pele, carne e de sementes das azeitonas. Além do óleo residual, este resíduo contém antioxidantes com grande valor para as indústrias de cosmética, alimentar e farmacêutica.

O objetivo deste trabalho é explorar o potencial do BA como fonte de antioxidantes, através da extração destes compostos com água subcrítica. Em paralelo, a hidrólise parcial deste resíduo também terá lugar, obtendo-se um licor rico em açúcar. Para efeitos de homogeneização, o BA foi divido em duas amostras, Amostra Fina (AF) e Amostra Grossa (AG). A amostra grossa era constituída maioritariamente por restos de caroço da azeitona, as partículas apresentavam diâmetros acima dos 1400 µm.

Antes da extração, ambas as amostras foram quimicamente caracterizadas. Verificou-se que a AF apresentava maior teor em lípidos (cerca de 55%) e em açucares solúveis (cerca de 5%). Por outro lado, a AG apresentava maiores teores em açucares insolúveis (cerca de 23%) e lignina (cerca de 60%). O teor de polifenóis obtido para as duas amostras foi baixo, menos de 1%, e o hidroxitirosol era o composto fenólico mais abundante.

A extração/hidrólise foi realizada apenas para AF, com fim a otimizar o processo e foram usadas as temperaturas de 140, 170 e 200 °C. Foram mantidas constantes, o caudal de água (10 mL/min) e a pressão (60 bar). Os melhores resultados foram obtidos a 200°C com um rendimento de compostos solúveis de 30%. Conseguiu-se extrair 20.87 mg/g de compostos fenólicos e uma recuperação de açúcares de 14% wt. face aos 18.12% existentes. Foi também determinada a atividade antioxidante dos extratos obtidos em cada ensaio, o melhor resultado foi nos extratos obtidos a 200 °C, tendo-se obtido um EC_{50}=32.83 mg/L. Foi também estudada a estabilidade dos compostos fenólicos, assim como da atividade antioxidante, e verificou-se, como seria de esperar, que ao longo do tempo, os compostos fenólicos vão-se degradando, e é mais acentuada quando estes compostos são expostos à luz e à temperatura ambiente.

Palavras-chave: agroindustriais; resíduos; bagaço da azeitona; água subcrítica; antioxidantes; compostos fenólicos

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BOD – Biological oxygen demand

C/N – Carbon/Nitrogen

Cd – Cadmium

CH_3OH – Methanol

CO – Carbon monoxide

COD – Chemical oxygen demand

Cr – Chromium

CT – Sample stored in **C**old **T**emperature

Da – Dalton

FAO – Food and Agriculture Organization of the United Nations

FAT – Sample stored in the **F**reezer at **R**oom **T**emperature

FS – Fine Sample

FT – Sample stored in the **F**reezer **T**emperature

GS – Gross Sample

ha – Hectare

Hg – Mercury

Kcal – Kilocalorie

Kg – Kilogram

Kton – Kilotonne

K_w – Ion product

L – Litre

LLE – Liquid-liquid extraction

m – Meter

m^3 – Cubic meter

MAE – Microwave-assisted extraction

MPa – Mega pascal

min – Minutes

NH_3 – Ammonia

OMW – Olive mill waste

OMWW – Olive mill wastewater

Pb – Lead

PLE – Pressurized liquid extraction

RT – Sample stored in **R**oom **T**emperatures

SCW – Subcritical water

SFE – Supercritical fluid extraction

SPE – Solid-phase extraction

T_c – Critical temperature

TN – Total nitrogen

TPC – Total phenolic compounds

ton – Tonne

TOC – Total organic carbon

TPOMW – Two-phase olive mill waste

UAE – Ultrasound-assisted extraction

WSC – Water soluble compounds

wt. – Weight

ε – Dielectric constant

μm – Micrometer

1 STATE OF THE ART

Over the decades the world population has been increasing and the trend is for the continuation of this growth. For example, in 2000 the world population was about 6.13 billion and today the world population is around 7.32 billion. With this increase in world population, there is an increase in consumption, at the same time there is great concern for environmental pollution, so as a result there is a concern and care for the recovery, recycling and upgrading of wastes or residues from the industry, for example the wastes from agro-industrial sector. [1], [2]

The biorefinery concept, which integrates all processes and equipment, with the objective of producing products with additional value as fuel, energy or chemicals, has recently started to incorporate biomass as a feedstock, thereby maximizing the value derived from the biomass. [3], [4]

Lignocellulosic biomass is one of many biomass feedstocks for biorefinery and it is considered to be a raw material of second generation used for the production of biofuels and chemicals. This raw material comes from many sources, for example forestry waste or agricultural waste, more specifically from agro-industrial wastes. [3]

1.1 Agro-Industry

According to FAO (*Food and Agriculture Organization of the United Nations*) the agro-industrial sector is defined as the subdivision of the manufacturing sector, where the main objective is the processing of raw materials and intermediate products derived from agriculture, fisheries and forestry.
In other words, the agro-industry consists of a number of manufacturing activities that have direct production relations between agriculture and manufacturing. [5], [6], [7]

So the increase in agro-industry results from the increased population and at the same time the increase in consumption. Other reason is the development of technology in areas such as agriculture, fishing and forestry, but also the technological development in the manufacture of products derived from these areas.

With this progress in last years a large amount of agro-industrial waste is produced in the word. That waste or residue have added value because have another uses or markets, such for production of

energy or valorization in other compounds that can be used in cosmetic, pharmaceutical or other areas. [8], [9]

1.1.1 Agro-Industrial wastes

Agro-industrial wastes are the most abundant, rich and renewable resources on earth, resulting from the treatment of a certain crop or animal product from agriculture, i.e materials similar to bagasse or oilseed cakes. [9]

Over the years, this biomass has been accumulated in large quantities which is an environmental problem, but also cause a devaluation of the biomass that has a potential value, because it can be processed to produce a countless products with added value, such as fuel or a variety of chemicals [9]. For example, in 2009 all over the world 150 Kton/year the residues from agriculture have been produced, that have potential for the production of other products. [10]

The agro-industrial waste can be divided and classified according to their physical characteristics. In this context we can divide the waste or residues into two categories: dry residues (such as straw) and wet residues (such as animal slurry). [9]

Dry waste includes parts of arable crops that were not used for the primary purpose as the production of food, feed or fiber. These residues can be divided into four classes according to their origin:

- Field and seed crop residues – that includes all materials left behind above the ground after harvesting, for example straw or stubble from barley;
- Fruit and nut crop residues – take account orchard pruning and brushes;
- Vegetable crop residues – include the vines and leaves that persist on the ground after harvesting;
- Nursery crop residues – consist the pruning's and trimmings taken from the plants in the course of their growth and in the preparation for market. [9]

On the other hand, the wet residues are wastes containing large water content and include animal slurry; farmyard manure and grass silage. [9]

The agro-industrial waste is mainly composed of lignocellulose that is compact structure, partially crystalline, which is composed of about 40-50% cellulose (linear and crystalline polysaccharide), 20-30% hemicellulose (branched non cellulosic and non-crystalline heteropolysaccharides), and 10-25% lignin (Figure 1.1). [9], [11]

Agro-industrial wastes also contain antioxidants, pigments, and vitamins with great interest in food, nutraceutical, and cosmetic sectors. [10]

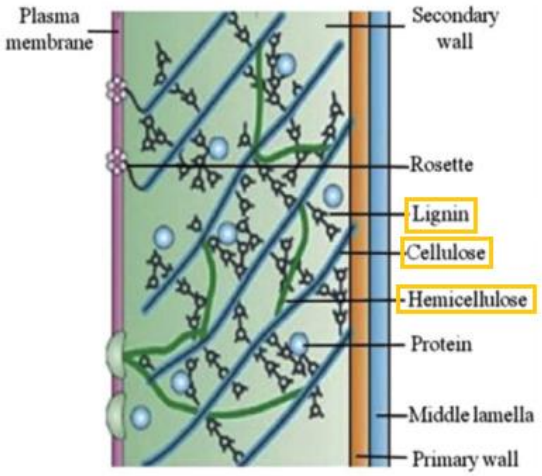

Figure 1.1 - Distribution of cellulose, hemicellulose and lignin in cell wall of lignocellulosic materials. Adapted from: [11]

But the composition of lignocellulosic materials depends on its source, whether it is derived from fruits waste, bagasse or grasses, Table 1.1. [11]

Table 1.1 - Percent composition of lignocellulose constituents in different lignocellulosic materials. Adapted from: [11]

Lignocellulosic material	Lignin (%)	Hemicellulose (%)	Cellulose (%)
Sugar cane bagasse	20	25	42
Sweet sorghum	21	27	45
Corn cobs	15	35	45
Rice straw	18	24	32.1
Banana waste	14	14.8	13.2
Bagasse	23.33	16.52	54.87

Cellulose is the main compound of agro-industry waste and is the most abundant biopolymer present in nature, and its function is to generate mechanical strength [11], [12]. It is composed of a linear polymer chain, which in turn contains a series of hydroglucose units that are attached by β-1-4 glycosidic linkages in glucan chains, Figure 1.2. [9]

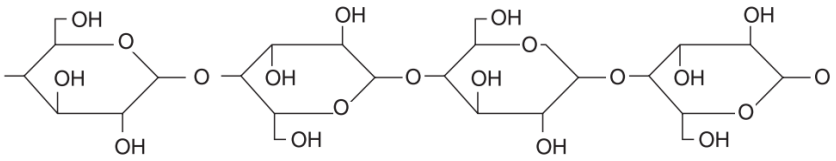

Figure 1.2 - Structure of cellulose. [9]

The cellulose molecules are attached by intermolecular hydrogen bonds in native state, but they have a highly predisposition to intra-molecular and intermolecular hydrogen bonds arrangements. This causes an increase in rigidity of cellulose, which makes very insoluble and making it resistant to most organic solvents. [11]

Hemicellulose is another main constituent of lignocellulose structure and the principal purpose is to behave as a coupling agent between lignin and cellulose. It's composed of several polysaccharides and constituted by C_5 and C_6 sugars (xylose, galactose, mannose, and arabinose), as well as other components such as acetic, glucuronic, and ferulic acids. [9], [13], [14]

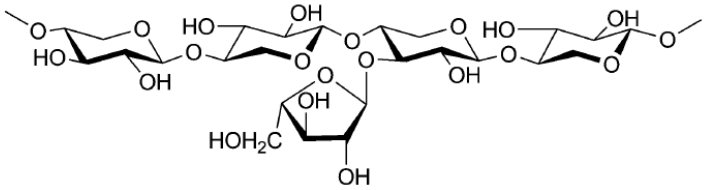

Figure 1.3 - Structure of hemicellulose. Adapted from: [13]

Hemicellulose is an amorphous polymer, which compared to the cellulose, is easier to hydrolyze in its sugar components. It is classified according to the primary sugar residue in its backbone, e.g., xylans, mannans, and glucans, with xylans and mannans being the most prevalent. [14]

Lignin is the smallest fraction in lignocellulose structure and is the most complex compound. Its main function is to act as a glue, thus filling the empty space between and around the cellulose and hemicellulose complexion with the polymers, and is consisted of phenyl-propane complex, methoxy groups and non-carbohydrate poly phenolic substance, that are biosynthesized in plants. [9], [11]

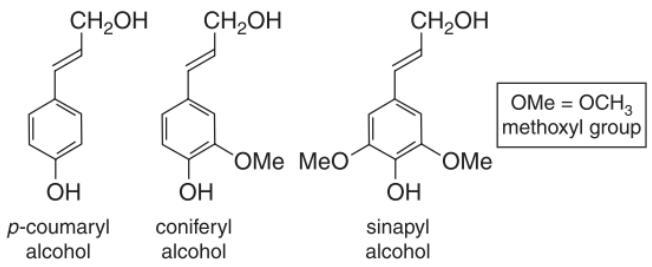

Figure 1.4 - C_6-C_3 phenypropanoid units. [9]

The C_6-C_3 phenylpropanoid units are the largest block in lignin structure, existing three units denoted as sinapyl alcohol, coniferyl alcohol and p-coumaryl alcohol, Figure 1.4. They are organized in a random and irregular three dimensional complex, which give strength and structure, conferring resistance to enzymatic degradation. [9], [11], [15]

1.1.2 Agro-industrial waste application

Agro-industrial waste is a promising feedstock that can be used in many ways because it is cheap and abundant. The advantage of using this type of waste is to reduce the deterioration of the environment, providing economic benefits and the fact that there is no competition with food production. The agro-industrial waste has a great potential value, because it can be processed to produce a number of valuable added products. [9], [10]

The principal application is the production of biofuels and bioenergy, because nowadays there is a great concern to reduce dependence on oil and to reduce greenhouse gas emissions which have a negative impact on environment. Also represents a positive impact on economy and on society. Other applications, is the extraction of fine chemicals, for example the antioxidants that can be used in cosmetic, and in farm for animal feed or as fertilizer. [10]

For example, in UK about 40% of wheat straw is sliced and returned to the soil, 30% used for animal bedding and feeding, and 30% is sold. Figure 1.5, shows a resume of lignocellulosic feedstock biorefinery and some applications. [9], [10]

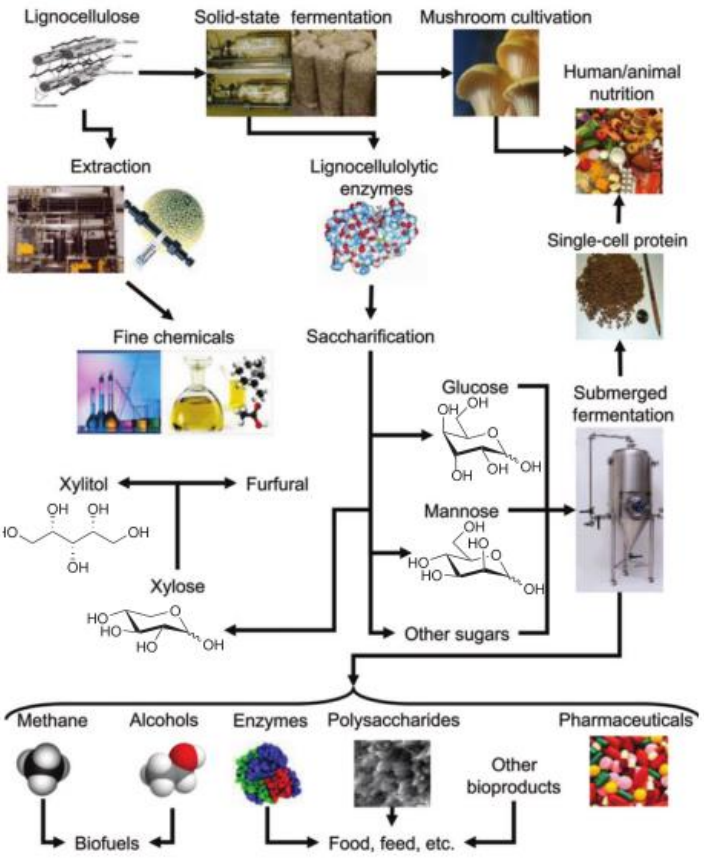

Figure 1.5 - Resume of lignocellulosic feedstock biorefinery and applications. [10]

1.1.3 Pre-treatment of Agro-industrial waste

As previously stated, agro-industrial waste consists of a lignocellulosic structure, which in turn is composed of cellulose, hemicellulose and lignin. To achieve these and other compounds obtained therefrom, such as glucose derived from cellulose which is present in large amounts in these waste, it is necessary to break the lignocellulosic structure. This breakage is made via a pre-treatment which is an important tool. For the choice of pre-treatment is necessary to take into account the purpose, the efficiency and the economic aspects. The pre-treatments that exist are: physical, chemical, biological (enzymatic) and combined. [9]

The Table 1.2 shows the various pre-treatment that are currently used for pre-treatment of agro-industrial waste.

Table 1.2 - Pre-treatment of agro-industrial waste. Adapted from: [9]

Pre-treatment	Examples	Effect of Pre-treatment
Physical	Irradiation	Depolymerization
	Hydrothermal	Hemicellulose hydrolysis, alteration in properties of cellulose and lignin
Chemical	NaOH, NH_3, H_2O_2, Preoxyformic acid	Lignin/hemicellulose degradation
	Peroxymonosulphate	Activates delignification
Biological	White-rot fungi (for example, *Bjerkendra adusta*)	Lignin degradation
Enzymatic	Lignin Peroxidases (LiP, MnP, laccase)	Selective lignin/hemicellulose degradation

Physical pre-treatment has mechanical and thermal methods with the objective to treat agro-industrial waste. But these methods have disadvantages, because they require a high energy input which increase the processing cost. [9]

Chemical pre-treatment is normally used to remove lignin, but can also remove the cellulose, present in the paper and pulp industries for paper production, by methods such as acid or alkali hydrolysis. The disadvantages of this pre-treatment are the requirement for corrosion-resistant apparatus, an effective washing approach and the ability for the safe disposal of used chemicals. [9]

Finally, in relation to the biological and enzymatic pre-treatments, as mentioned before the lignin is part of the lignocellulosic structure and is associated with cellulose and hemicellulose in the cell wall.

The pre-treatment is important to obtain a good degree of fermentable sugars from agro-industrial wastes. The pre-treatment will change the cell structure of cellulose to make them more accessible for enzymes to convert the carbohydrate polymers into fermentable sugars. [9]

For biological pre-treatments it has been commonly used white-rot fungi and certain bacteria, that are used to remove lignin from lignin degradation, but this is unselective comparative to lignin which is degraded only to obtain the more readily metabolized cellulose and/or hemicellulose. [9]

In this work it is used an agro-industrial waste from olive oil extraction, more precisely, the olive pomace which is a by-product of this industry. This residue consists of antioxidants with potential added value for the cosmetics, food and pharmaceutical industries.

1.2 Olive Tree

"The origin of the olive tree is lost in time, coinciding and mingling with the expansion of the Mediterranean civilizations which for centuries governed the destiny of mankind and left their imprint on Western culture." Cited from: [16]

The olive tree, whose scientific name is *Olea europaea L.*, is a small evergreen tree that averages 3 to 5 m in height and grows practically around the Mediterranean Sea. This simple tree has been praised over the centuries by every civilization, who inhabited and inhabit the countries around the Mediterranean Sea. [17]

For 8000 years, the olive tree has held a special place in human culture, crossing many civilizations and religions. In the days of ancient Greece, the winners of the Olympic Games were crowned with a crown of olive branches and in the era of the Romans, they used them to rituals. [18]

Figure 1.6 - Olive tree or Olea europaea L. [19]

The olive branches in religion, both in the Hebrew religion as the Christian, have a symbolic meaning, being associated with peace and brotherhood. In the Koran one can also see references to the olive tree. [18]

In the Table 1.3, shows some of the varieties of olive trees as well as its origins. Each variety of olive trees, gives olives different sizes as well as the specific features.

Table 1.3 - Some varieties of olives trees. Adapted from: [20]

Type	Origin	Characteristics
Arbequina	Spain (Catalonia)	Small tree and give small fruit but very small pit. Relatively resistant to cold.
Arbosana	Spain	Semi-dwarf cultivar. Give fruit with medium-size.
Chemlali	Tunisia	Large and vigorous tree. Give fruit with small size.
Coratina	Italy	Vigorous. Gives fruits with large size and needs a pollinator.

Cultivating olive trees in general takes time, normally the first crop is expected after 8 to 10 years, but nowadays there are some varieties that give good olive in a shorter period of time (4-6 years). [17] The trees are characterized by having small leaves (pale green above and silvery below) and in May, the tree bloom small and creamy white flowers. In October, the olive fruit can be harvest, because is when the fruit is mature. [17]

Because the biennial cycle affects the olive tree yield, every two years are generated more olive oil and waste, example in one year it grows and the other year gives more fruits. The other reason is the weather conditions in the Mediterranean basin. [17]

Others factors that can affect the olive tree yield are the climate and the soil. Normally, the olive trees grow in a dry and subtropical climate, but can also grow in extreme environmental conditions, such as drought and high temperatures. About the soil, trees require a sandy soil, but they are adaptable to a wide variety of different soil types. [21]

The fertilizer and the way to do this fertilize, can also affect the olive tree yield.

A special feature of the olive tree is that it is a very resistant tree, it can resist drought, disease or fires, which gives longevity, for example, in Europe most of the areas of olive tree are old, wherein 2.7 million ha (59%) of total area are at least 50 years old, Figure 1.7. [22]

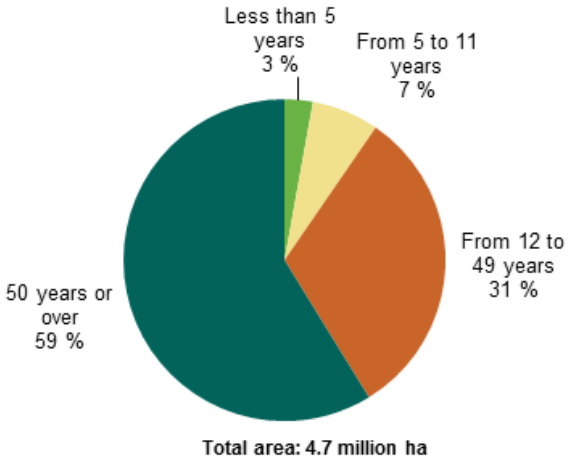

Figure 1.7 - Area of olive trees by age classes in Europe, 2012. [22]

Olive oil is not the only product of olive tree, but it is the main product. Olive tree gives a number of other by-products or residues as biomass from pruning, olive stones, pomace oil and pomace residues, and wastewater, Figure 1.8. [23]

These by-products, nowadays, are reused in bio refineries, to reduce the amount of waste, which is an environmental concern.

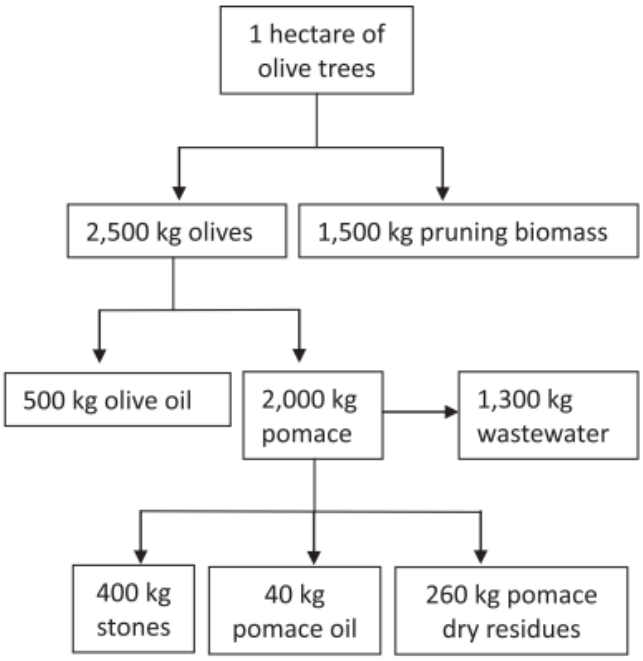

Figure 1.8 - Average mass balance olive trees corresponding to Spanish typical cultivation procedure. [23]

In 2012, the area occupied by olive trees groves across Europe, Figure 1.9, was about 4.65 million ha, wherein the production is concentrated in Mediterranean area, where over three quarters of the total EU area of olive tree is from Spain (53%) and from Italy (24%). [22]

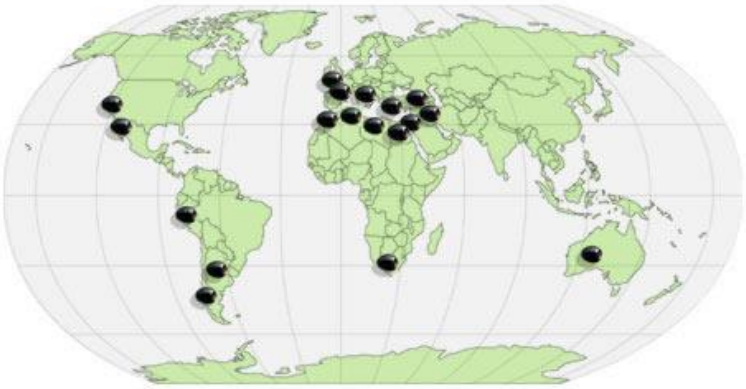

Figure 1.9 - Area occupied by olive trees. [21]

The area occupied in Portugal represents 7% of the total EU area, moreover the area occupied in Spain is about 53%, which can see in graphic of Figure 1.10. [22]

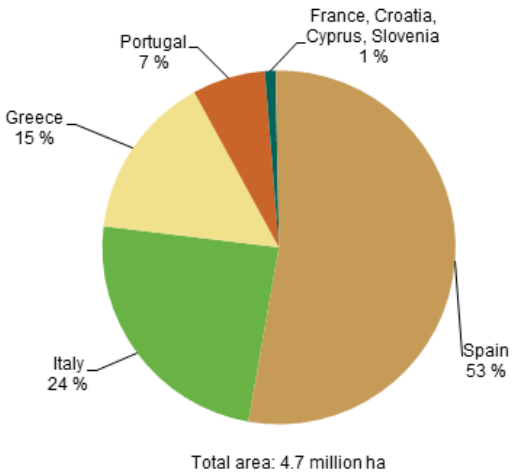

Figure 1.10 - Percentage of area occupied in Europe, 2012. [22]

Portugal

Portugal is the fourth largest producer off olive oil, with 430 Kton of olives in 2012 [24], and in same year the area of olive tree has the 312 276,07 ha. [25] As we can see in Figure 1.11, the distribution of olive trees is predominant in Ribatejo, Norte Alentejano and Beira-Interior.

Figure 1.11 - Distribution of olive trees in Portugal. [26]

What makes Portugal the perfect country for production of olive trees, is the climate, because:

- Have low chance in the occurrence of hail storms, particularly when out of season and that is able to destroy entirely production;
- The thermal variations are not too prominent and because of that, the probability of fungal diseases in olive plantations are reduced.

But the most important factor that contributes for the development of olive trees is the rainfall rate, that in Portugal is very high. This means that, when the soil is wet the olive trees yield increases, albeit not in excess. [27]

Like all countries, different climates give different olives trees, and Portugal is no different. So in Portugal there are about 30 varieties of native olive tree, adapted to its climate, and 4 of 30 existing varieties are the most representative in Portugal, and they are: *Cobrançosa*, *Cordovil*, *Galega* and *Verdeal*. [27]

1.3 Olive

The olive tree gives a fruit that can be harvested in October and is called olive. This fruit is a drupe and composed of four parts, Figure 1.12: [28], [29]

1) The pulp or flesh, as known as mesocarp, representing 70-90% of the entire fruit, and is responsible for the source of all the constituents;

2) Stone wall or woody endocarp is 9-27% of the olive weight and surrounds the seed;
3) The skin or also termed as epicarp representing 1-3% of the drupe weight and is constituted of chlorophylls, carotenoids and anthocyanins which are responsible for the color;
4) Seed, which represents 2-3% of the entire fruit.

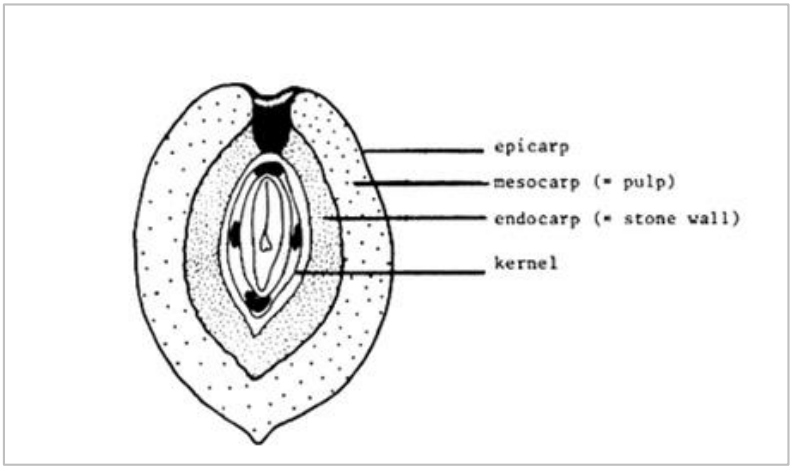

Figure 1.12 - The olive cross-section. [30]

It's a fruit that cannot be consumed straight from the tree, because it has *oleuropein* that has a bitter taste. It is also composed of a low sugar content (2.6-6%) and a high oil content (12-30%), that depend on the time of year and the variety of the fruit. [31]

In Table 1.4, we can see the composition of constituents of olives, as we can see the fruit is most constituted of oil and water.

Table 1.4 - Composition of olives. Adapted from: [16]

Constituents	Pulp (wt. %)	Stone wall (wt. %)	Seed (wt. %)
Water	50 – 60	9.3	30
Oil	15 – 30	0.7	27.3
Compounds containing nitrogen	2 – 5	3.4	10.2
Sugar	3 – 7.5	41	26.6
Cellulose	3 – 6	38	1.9
Minerals	1 – 2	4.1	1.5
Polyphenols	2 – 2.25	0.1	0.5 – 1
Others	–	3.4	24

The olive may have two purposes depending the species of olive, but its principal purpose is for production of olive oil, the other is for direct consumption as table olive. For example, in Portugal exist 30 varieties of olive, that can use for production of oil or direct consume or both, depending on the characteristics of the olive, Table 1.5 and Figure 1.13.

Table 1.5 - Types and destiny of olives in Portugal. Adapted from: [32], [33]

Type	Destiny
Carrasquenha	Olive oil and table olives
Cobrançosa	Olive oil
***Cordovil* from Castelo Branco**	Olive oil and table olives
Galega	Olive oil and table olives
Maçanilha Algarvia	Olive oil and table olives
Redondil	Olive oil and table olives
Picual	Olive oil
Verdeal	Olive oil and table olives
Madural	Olive oil

Figure 1.13 - Types of olives in Portugal. Adapted from: [32]

In Portugal most olives have high productivity and each with their performance characteristics, each olive gives oil with different organoleptic characteristics. For example, the *Cobrançosa* olive (Figure 1.13) has high productivity with yield high olive oil. The olive oil produced from that type of olive is balanced and can have a sweet and smooth profile or medium fruity, subtly bitter and spicy, depending on when it is harvested. On the other and the *Galega* type (Figure 1.13) have high productivity but low oil yield; the olive oil produced from this type is smooth and sweet. [34]

Nowadays olives, as olive oil, have been viewed as a very healthy food. Besides providing energy, they are composed of significant amounts of antioxidants, minerals, phytol-sterols, vitamins and is an excellent source of fat because of the monounsaturated fatty acids, and they are beneficial in the control of heart disease, the brain function and the skin, decrease the risk of cancer and might help to prevent strokes. [35]–[37]

1.4 Olive oil production

In Europe the olive oil production is an important agricultural sector mostly located in countries and areas bordering the Mediterranean Sea, and represented 80% of the world production of olive oil and also the most consumer representing 70% if the consumption in the world. [38]

In 2010 the olive oil production is estimated to have reached 2.12 million tones and about 85% off that production comes from Spain, Italy and Greece. [39]

In the Table 1.6, we can see main oil-producing and consuming countries in 2005.

Table 1.6 - Main oil-producing and consuming countries in 2005. Adapted from: [37]

Countries	World production (%)	World consumption (%)
Portugal	1	2
Spain	36	20
Italy	25	30
Greece	18	9
Turkey	5	2
United States	1	8
Syria	4	3

Nowadays the production of olive oil takes into account environmental aspects on the way to have a reduction of the waste and the low-energy and chemicals-free industrial process. [17]

1.4.1 Olive oil extraction process

The process for separating and collecting oil from the olives is denominated as olive oil extraction. The quality and equality of oil depends on the quality of olives itself, the harvest time and also the extraction method.

Olive oil extraction consists of different steps, such as olive washing, grinding, beating and the extraction itself, which is the most important phase and the basic stage of the whole process.

The extraction of oil from olive can happen in two different processes that are commonly used:

i. The Batch process or also known as traditional process or press process;
ii. The continuous process, or also known as centrifugation. [17], [29], [38]

The olive oil extraction by the traditional way, is made using press technology, used for several centuries with minor alterations. In this process the olives are washed, crushed, and mashed with the addition of hot water. The resulting paste is then pressed to remove the oil. The liquid from the press residue comprises a mixture of olive juice and water added and contains residual oil. Finally, the oil is separated from water by decantation or centrifugation vertical. Despite having small quantity of water used during the extraction process, the produced waste water, also called as olive-mill wastewater or OMWW, is highly polluted. [17]

In the continuous process a horizontal centrifuge is used, that allows continuous operation. Compared to the extraction made by the traditional process, the oil extraction made by the continuous process is advantageous because the cost of labor is lower, there is less need for space, better quality due to the elimination of mat flavor, improved process control and higher ease of automation. But the main advantage is the increased production compared to the traditional method. The major disadvantage of this process is the high capital. This process can be divided in two sub processes, depending on separation method used:

a. The 2-phase decanter process;
b. The 3-phase decanter process. [17], [29], [38]

The 2-phase decanter process appeared in the 90's, as being a clean and green process for the extraction of oil, because with this process practically eliminates the liquid effluent produced, since there

is no water addition and there is only one residue resulting from the process, in other words, the liquid and solid residue are originated together in the form of a mud, which is very moist and contains traces of oil, that sludge is the pomace or "*alperujo*" as designated in Spain.

The difference between 2-phase and 3-phase decantation process is the number of streams, consequently, the phase of the product /waste obtained in each chain.

In the 2-phase decantation process, we obtain two streams, one which is the product, the oil, which is a liquid phase and a stream of residue, which as stated above as the "*alperujo*", which is a solid residue with a high moisture content. On the other hand, the 3-phase decantation process, we obtain 3 streams, two streams that are in the liquid phase, which corresponds to the oil and other wastewaters and another stream which is a solid residue, which designates as olive cake or olive pomace, Figure 1.14. [17], [29], [38], [40]

Summarizing, what differs these processes (continuous and traditional) is the amount of water used in the process and the amount and composition of waste streams which may vary considerably, but the yield of olive tree is basically the same independently the process used. Figure 1.14 gives a resume for each process, where can see the differences between de processes. [16]

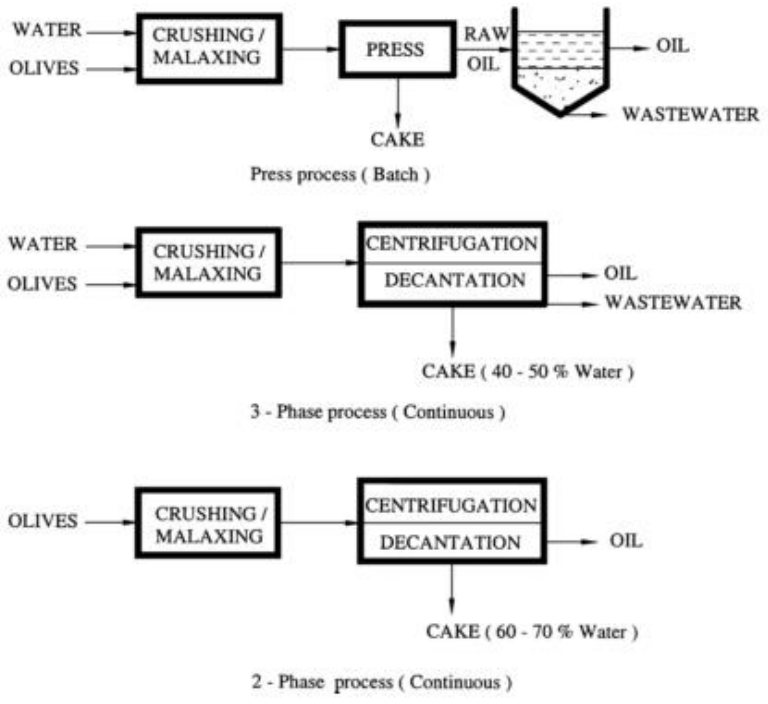

Figure 1.14 - Olive oil extraction process. [17]

The Table 1.7 shows an estimative of quantities of waste streams from the three types of olive oil production process and the main parameters for different oil production techniques.

Table 1.7 - Estimated quantities of Input-Output from the Three Types of Olive Oil Production Processes. Adapted from: [17], [41]

Production process	Input	Amount of input	Output	Amount of output
Traditional press process	Olives	1 ton	Oil	≈ 200 Kg
	Wash water	0.1 – 0.12 m^3	Solid waste (25% water + 6% oil)	≈ 400 Kg
			Wastewater (88% water + solids and oil)	≈ 400 - 600 L
Three-phase process	Olives	1 ton	Oil	200 Kg
	Wash water	0.1 – 0.12 m^3	Solid waste (50% water + 4% oil)	500 – 600 Kg
	Fresh water for decanter	0.5 – 1 m^3	Wastewater (94% water+ 1% oil)	1000 – 1200 L
	Water to polish the impure oil	≈ 10 L		
Two-phase process	Olives	1 ton	Oil	200 Kg
	Wash water	0.1 – 0.12 m^3	Solid + water waste (60% water + 3% oil)	800 – 950 Kg

The amounts of wastewater produced by 3-stage process is three times more than the traditional process. Although in the traditional process the amount of wastewater is less than in 3-phase process, they are more concentrated in pollutants. On the other hand, continuous process of two phases is called "ecological process" because unlike the other processes uses less amounts of water. The wastewater from this process are in smaller quantities and result of washing water, containing so less polluting compared to the other two processes. [17]

Currently the oil producing countries has been slow to implement the process of two phases for oil production, because this process minimizes the high costs of wastewater treatment. Furthermore, as mentioned above, with this process there is an economization of water and energy, 80% and 20%, respectively. In terms of investment costs this process requires less cost by about 25% compared to the 3

stage process. This technology produces in addition to sewage, a solid-liquid residue, called olive pomace or alperujo, which contains about 60% water and 3% oil. [17]

1.4.2 Olive mill wastes

Olive oil extraction is characterized by significant amounts of by-products, namely as olive mill wastes (OMW). In the last years, the demand and the consumption of olive oil has been gradually increasing worldwide and at the same time the amount of OMW is increasing, this causes an increase in environmental pollution, a problem that has been observed especially in the Mediterranean region. Because of that, the olive oil producers have searched for environmental sound, economic and viable solutions for handling and disposing of OMW, but currently they've been facing a serious challenge to find that solution. [17]

The olive oil wastes can be solid (for example, olive stones), liquid (as olive mill wastewater) or both (as olive pomace), containing high concentration of organic and phenolic compounds and they are composed of numerous compounds that aren't easily degradable and have acidic character (3-5.9 pH). The composition of wastes from oil extraction depends on various factors such as the time of harvest, the degree of ripening, the variety of olives, the use of pesticides and/or fertilizers and the type of extraction process. Weather conditions and the type of soil for cultivation also influence the composition of waste in qualitative and quantitative terms. [17], [38], [41]

The OMWW or olive mill wastewater, is the main source of environmental pollution in the olive oil-producing countries, and it is one of many wastes obtained in the extraction of oil. This waste is characterized for is high organic (COD/BOD ratio between 2.5 and 5) content, by having a slightly acid pH, high content of polyphenols and solid matter. In Table 1.8, shows the differences between chemical-physical characteristics from discontinuous and continuous process for OMWW. [17], [38], [41]

The treatment of this waste is problematic and expensive, because of the presence of organic substances, which interferes in the balance soil-air/air-water, which causes a reduction of fertility on the soil. The presence of polyphenols causes phytotoxic effects on soil, which cause problems in the treatment too. For this reason, the 2-phase decanter process was invented for decrease the amounts of this waste, because less water is used in the process. Other's methods have been tried to reduce the amounts of OMWW, but with time these methods have gradually become less viable for OMWW disposal. [28], [41]

Table 1.8 - Chemical-physical characteristics from OMWW. Adapted from: [41]

Parameters	Discontinuous process	Continuous process
pH	4.5 – 5	4.5 – 5
COD (g/L)	125	50
BOD (g/L)	90	40
Carbohydrates (%)	4.5	1.5
Tannins (%)	1.5	0.37
Phenolic compounds (%)	1.7	0.63
Suspended solids (%)	0.1	0.9
Total nitrogen (%)	1.8	0.3

As previously stated, 2-phase decanter process is an ecological method because reduce significantly the amounts of wastewater. The residue produced by this method will be a solid and very humid by-product designated as olive pomace, or "*alperujo*" as it is called in Spain. [28]

So independently of the process, the OMWW produced contain multiple nutrients that can be further developed and applied in many areas, Figure 1.15. [42]

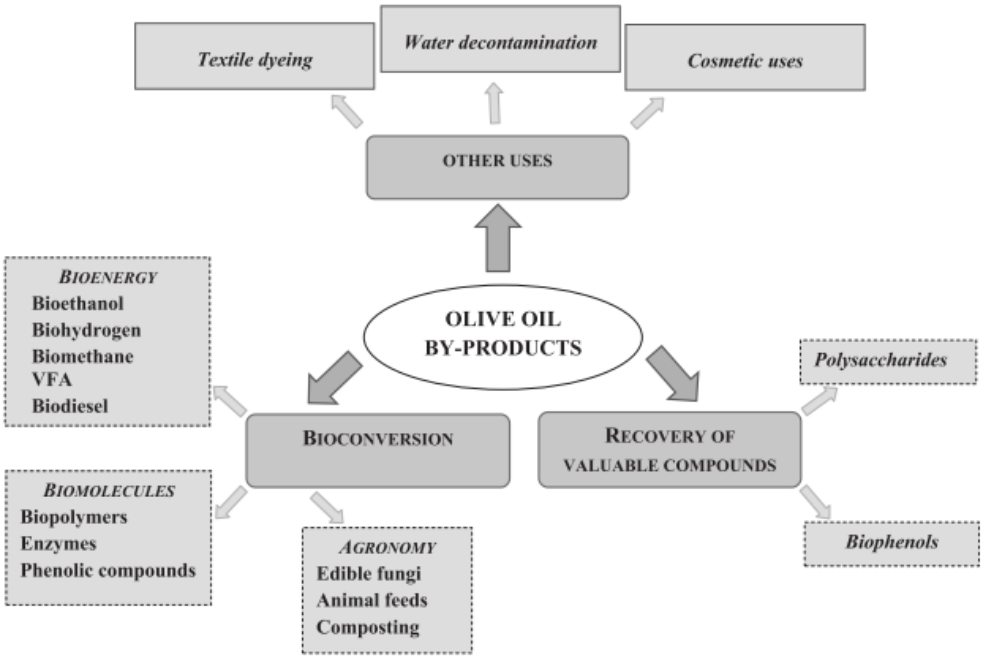

Figure 1.15 - Applications for OMWW. [42]

The principal objective of this work is to exploit the potential of olive pomace or "*alperujo*" as a source of antioxidants and other components with additional value that can be used in areas such as cosmetic or pharmaceutical.

1.5 Olive pomace

Olive pomace also called "*alperujo*" or "*alpeorujo*" (sometimes referred as olive wet husk, olive wet pomace, olive wet cake or two-phase olive mill waste, TPOMW) is the by-product of the extraction of olive oil through the 2-phase decanter process. It is characterized by being a very humid sludge, containing solid particles and oil traces, and is basically a mixture of skin, flesh and seed from olives. This waste has a strong odor and a pasty texture making it difficult to transport and handling. [40], [43], [44]

The "*alperujo"* has a high moisture content, a slightly acidic pH, is very rich in organic material (as lignin or fats), and it is also rich in phenolic compounds, Table 1.9 and Table 1.10. Concentration of heavy metals is lower than 1 mg/Kg for Pb, Cd, Cr and Hg. [28], [29], [43], [44]

Table 1.9 - Main components of the organic fraction of the "alperujo" with respect to the total organic matter content. Adapted from: [28]

Parameters	Percentage Range (wt. %)
Lignin	35 – 60
Hemicellulose	29 – 45
Cellulose	15 – 27
Fats	8 – 21
Protein	5 – 12
Water soluble carbohydrates	1 – 18
Water soluble phenols	1 – 3

Table 1.10 - Main characteristics of the "alperujo". Adapted from: [28]

Parameters	Range
Moisture (%)	56 – 75
pH	4.86 – 6.45
Ash (g Kg^{-1})	24.0 – 151.1
TOC (g Kg^{-1})	495.0 – 539.2
C/N ratio	28.2 – 72.9
TN (g Kg^{-1})	7.0 – 18.4

The chemical composition is variable depending on multiple factors as the variety of olives, culture conditions, harvest time, processing methods and extraction yield, but in the end it's relativity similar. [29], [43], [44]

In Table 1.9, shows the main constituents of "*alperujo*" are lignin, hemicellulose and cellulose, which is main organic components. The alperujo is also constituted by soluble carbohydrates, as mannitol, sucrose and fructose, and fats, which are very good constituents to occur growth of microorganisms. Other compounds are present in this substrate with high value, as polyphenols, for example, oleuropein, hydroxytyrosol and caffeic acid, that can be applied as cosmetic or pharmacological agent. [43]–[45]

1.5.1 Applications

The "*alperujo*" management is very expensive, because it requires specific facilities, for example, storage tanks with special valves, because of that this residue has become a serious problem for olive mills. So over the years there have been studies for the "*alperujo"* valorization in order to be used in multiple applications providing an economic advantage. [29], [44]

I. Extraction of residual oil

Alperujo contains residual oil, which may be recovered through a 2nd extraction solvent, using for example hexane, but to perform the extraction it's necessary that the residue is dried. From this extraction results another residue "*orujillo*" or olive cake which subsequently has been able to be used as fuel.

The high operating costs and the need for large amounts of energy to effect drying, leads to a lower demand of this residual oil. [29], [44], [46]

II. Production of bioenergy and biofuels

The production of bioenergy and biofuels through agro-industrial waste, contributes to the preservation of the environment, generating energy and fuels that are renewable and clean. The waste resulting from the oil extraction are no exception. For example, the residue resulting from the oil extraction of the alperujo is a good source of thermal or electric energy, because it has a great calorific value of about 400 Kcal/Kg. The thermal or electrical energy is obtained by combustion of residue. The main drawbacks of this method is the large production of ash from the combustion and the total energy recovery is low, since much of the energy obtained is used for drying the residue. [43], [44]

Currently there is an alternative method for combustion called biomass gasification. It is a physical-chemical method, which transforms the solid biomass into synthesis gas, or syngas. The synthesis gas is a mixture consisting primarily of CO, H_2 and CO_2 used for the production of fuel through its transformation into methanol and subsequently into dimethyl ether, or for obtaining important chemicals such as NH_3 and CH_3OH. The alperujo has a calorific value of 3500 Kcal/Kg and has potential because it contains a high moisture content, low ash and low density, which are the main characteristics of a fuel biomass. [38], [44], [47]

The alperujo can also be used for biodiesel production, because it contains a high oil content. The main fatty acid present in the residue, oleic acid (C18:1) is the preferred fatty acid for the production of biodiesel, in addition to containing significant amounts of linoleic acid (C18:2) and palmitic acid (C16:0). [48]

III. Application in agriculture

The "*alperujo*" can be used in agriculture as organic fertilizer as a result of the composting process, because it contains high content of organic matter and micronutrients.

The composting process is a process of valorization of organic matter and consists in the decomposition of waste by aerobic organisms. The biodegradability of organic matter follows the following order: carbohydrates > hemicellulose > cellulose > lignin (lower degradability).

This residue is easily degraded because it contains a low content of carbohydrates, but that degradation can be difficult due to the large amount of lignin in the waste (which makes enzymes and microorganisms have difficulty making this degradation) and the fact that this waste has also a very high moisture content, about 65% water. The C/N ratio is also an important factor to take into account when it comes to composting, because the higher the ratio the more difficult is the process. The ideal ratio for

composting is between 25–35. The "*alperujo*" has a high C/N ratio (28–73) that can be easily corrected by nitrogen supplements. Before being used as fertilizer and applied directly to the soil, "*alperujo*" needs to be treated via bioremediation, aiming to degrade or polymerize the phenolic compounds, eliminating the toxicity because large phenols content originates symptoms of phytotoxicity[1] on crops. Despite this, composting and subsequently the application as fertilizer, it proves to be a practical low cost and a strategy for reducing this residue and thereby reducing the danger to the environment. [28], [43], [44], [46], [48]

IV. Extraction of value compounds

A large number of scientific articles demonstrate *"alperujo"* or olive pomace as a low cost substrate with great potential for the production of valuable compounds.

The olives are constituted by a wide variety of phenolic compounds of which less than 1% of these compounds are found in olive oil and the remaining 98% are in the olive pomace. The major phenolic compounds are hydroxytyrosol, tyrosol, oleuropein and caffeic acid. These compounds have great interest since they can be applied in the pharmaceutical industry, cosmetics and even in the food industry.

The cell walls of olive pulp containing about one third of arabinose-rich pectic polysaccharides, therefore, has emerged another alternative to the alperujo, which is the extraction of pectins[2], which are natural hydrocolloids, widely used in the food industry as gelling agents, stabilizers, and emulsifiers. [38], [43], [44], [49]

1.6 Phenolic compounds

In recent decades, society has been characterized by poor dietary habits, which lead to an increase in chronic diseases such as obesity or cardiovascular diseases. But epidemiologic and biochemical studies have shown that regular consumption of fruit and vegetables cause a reduction in heart disease, as well as common cancer and other degenerative diseases. This can be explained by the presence of antioxidants in fruit and vegetables. Of all the antioxidants, the most common and known antioxidants are the vitamins, such as, ascorbic acid (typically known as vitamin C), α-tocopherol (Vitamin E) and β-carotene

[1] Phytotoxicity is when a substance inhibits seed germination, root growth or development of plants. [108]

[2] Pectins are complex polysaccharides consist of 1,4-linked-α-D-galactosyluronic acid residues that are interrupted by (1→2)-α-L-rhamnose residues transporting sugar side chains, typically galactose and arabinose. [49]

(provitamin A). In recent studies it has been proved that phenolic compounds found in plants are phytochemicals with high antioxidant properties. [50]–[52]

Phenolic compound or polyphenols are widely distributed in plants and correspond to the main class secondary metabolites, that derivatives of biological pathway, such as phosphate, pentose, shikimate and phenylpropanoids pathways. These compounds play an important role in physiology and morphology of plant life, contributing to the growth and reproduction of plants. They are crucial in structural terms, and can participate in support or protection functions, against pathogens and predators. Phenolic compounds contribute to the organoleptic properties in terms of color, aroma and taste (since they are involved in astringent and bitter tastes) and also contribute to the nutritional quality of plants. [51]–[53]

The phenolic composition may range depending on the stage of maturity, exposure to light, temperature and in cultivation. When the concentration of phenolic compounds is low, they can act as an antioxidant, protecting the food against oxidative deterioration, but in high concentrations, they can interact with the proteins, carbohydrates and minerals. [51]–[53]

The polyphenols are important compounds because of their positive contribution to health because exhibit a wide range of physiological properties such as anti-allergic, antiatherogenic, anti-inflammatory, antimicrobial, antithrombotic, cardioprotective and vasodilatory effect. Furthermore, they retard or inhibit the auto-oxidation by acting as radical scavengers, and are therefore, essential antioxidants that protect against the propagation of oxidative chain. [51]–[55]

In recent time, the interest in natural antioxidants has been increasing in an attempt to reduce the consumption of synthetic antioxidants (for example: butylated hydroxytoluene, BHT, or butylated hydroxyanisole, BHA) because they are suspected of causing adverse effects to human health, because the ingestion of higher concentrations of these antioxidants may cause mutagenic and carcinogenic modifications and lead to chronic side effects. [56], [57]

The structure of these compound has an aromatic ring been able to have one or more hydroxyl groups, which can give a wide range of phenolic molecules from simple molecules to highly polymerized phenolic compounds, Figure 1.16. Most of the phenolic compounds occur naturally and may find present as conjugates with mono- and polysaccharides linked to one or more of the phenolic groups, and may also occur as functional derivatives of these esters and methyl esters. [51]

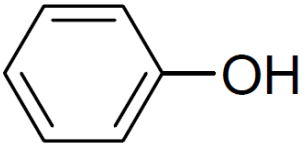

Figure 1.16 - Basic structure of a phenolic compound. Adapted from: [51]

Phenolic compounds can be divided into different classes depending on the chemical structure, i.e. they can be classified taking into account the number of phenolic rings that they contain and on basis of structural elements that bind these rings to one another. In this context, the main classes are phenolic acids, flavonoids, tannins, lignans and stilbenes. [52], [53], [58]

Phenolic acids are found in abundance in foods and can be divided into two subclasses: derivatives of benzoic acid, designated as hydroxybenzoic acids, and derivatives of cinnamic acid, known as hydroxycinnamic acids, Figure 1.17. [52], [58]

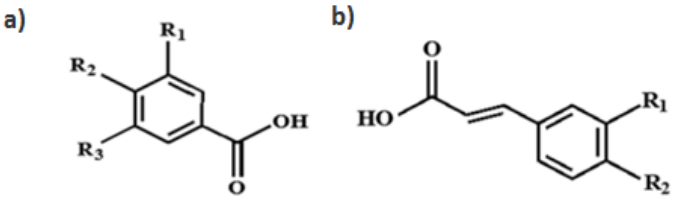

Figure 1.17 - Basic structure of phenolic acids: a) Hydroxybenzoic acids; b) Hydroxycinnamic acids. Adapted from: [58]

The hydroxybenzoic acids have in common the structure of C_1-C_6 and include gallic, *p*-hydroxybenzoic, protocatechuic, vanillic and syringic acids, Figure 1.18. In edible plants, hydroxybenzoic acid content is generally low, except for certain red fruits. The hydroxycinnamic acids are aromatic compounds with a side chain of three carbons displaying a C_3-C_6 structure and acids include caffeic, ferulic, sinapic and *p*-coumaric acids, Figure 1.18. Compared to hydroxybenzoic acids, the hydroxycinnamic acids are more common in food. [52], [58]

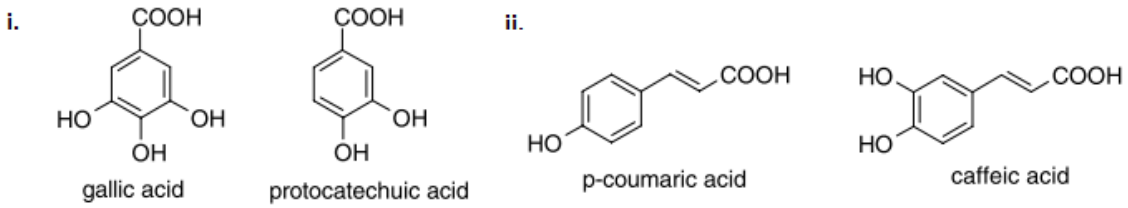

Figure 1.18 - Examples of phenolic acids: i - hidroxybenzoic acids; ii - hydroxycinnamic acids. Adapted from: [52]

Flavonoids are the largest group of phenolic compounds present in plants with low molecular weight and consist of 15 carbon atoms arranged in a C_6-C_3-C_6 configuration. The structure is basically formed by two aromatic rings A and B, joined by a 3-carbon bridge, commonly in the form of a heterocyclic ring C, Figure 1.19. [52]

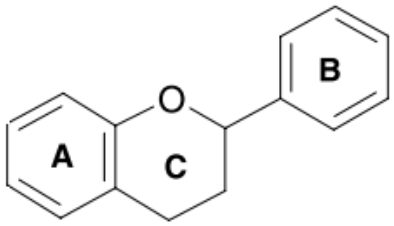

Figure 1.19 - Basic structure of flavonoids. Adapted from: [52]

They can be divided into seven subclasses based on the variation of the heterocyclic type involved: flavones, flavonols, flavanones, flavonols (catechins), anthocyanins, isoflavones and chalcones (Figure 1.20). The variation in the number and arrangement of hydroxyl groups, as well as the nature and extent of alkylation and/or glycosylation of these groups leads to individual differences within each group. [52], [53], [58]

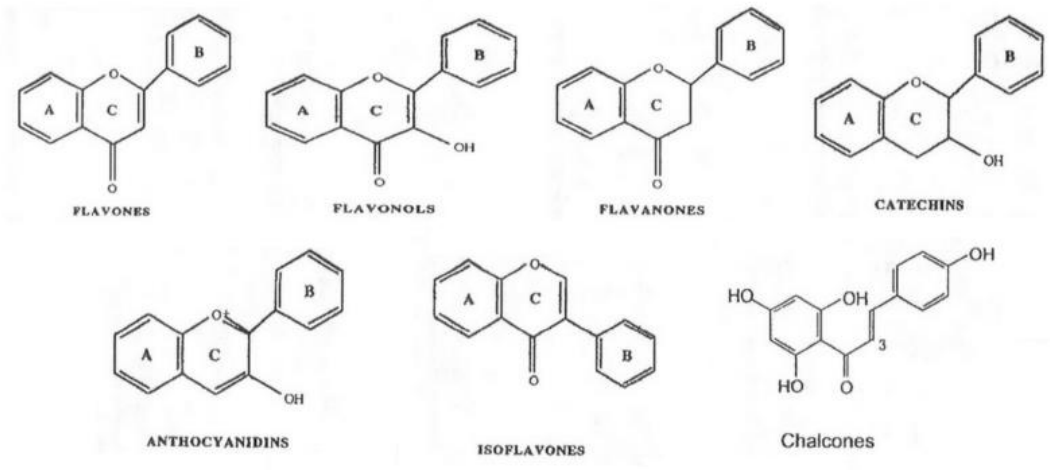

Figure 1.20 - Generic chemical structure of subclasses of flavonoids. Adapted from: [53]

The flavonoids can be found in foods and are normally glycosylated, wherein the linked sugar is often glucose and rhamnose, but can also be galactose, arabinose, xylose, glucuronic acid, and other sugars. In this context glycosylation influences the physical and biological properties of flavonoids. [53]

The tannins are the third most significant group of the class of polyphenols and are defined to be compounds with a molecular weight range between 500 Da and 3000 Da, found in complexes with alkaloids, polysaccharides and proteins. They are a group of water soluble polyphenols capable of binding

proteins, which could give complex tannin-protein which might be insoluble or soluble, which allows making complexes with polysaccharides (cellulose, hemicellulose and pectin), and nucleic acids, steroids, alkaloids and saponins. They may be located in plant leaves, fruit, legumes, wood and roots, more specifically in the tissues in the vacuoles. [52], [59], [60]

Tannins may be divided into two main groups according to their chemical structure and properties: hydrolysable tannins and condensed tannins (Figure 1.21). The hydrolysable tannins are esters of gallic acid (gallotannins and ellagitannins). Their chemical structure contains a carbohydrate, usually D-glucose, as a central core. On the other hand, the condensed tannins are constituted by flavonoid units (flavan-3-ol) linked by carbon-carbon bonds and they are usually found in legumes. In plants concentrations of hydrolysable tannins are lower than the condensed tannin. [52], [59], [60]

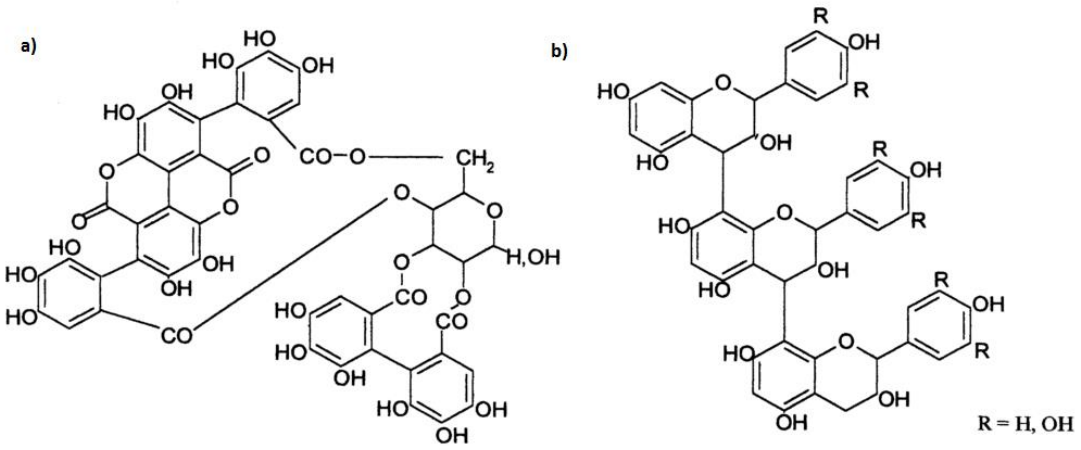

Figure 1.21 - Basic chemical structure of tannins: a) Hydrolysable tannins; b) Condensed tannins. Adapted from: [61]

Lignans are secondary metabolites of vascular plants with beneficial properties and wide variety of physiological functions. They are compounds derived from diphenolic combination of two C_6-C_3 units phenylpropanoids connected by its central carbon (C_8), Figure 1.22. They can be found in edible plants which occur free or bound to sugars and the plant lignans most commonly found in foods are lariciresinol, matairesinol, pinoresinol and secoisolariciresinol. [62], [63]

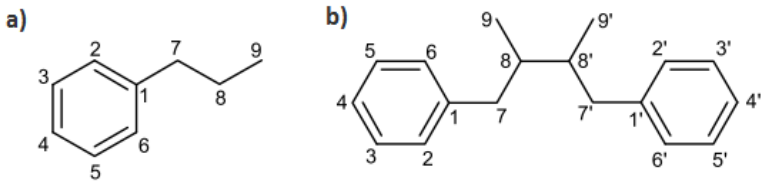

Figure 1.22 - a) Phenylpropanoid unit; b) Basic structure of lignans. Adapted from: [62]

The stilbenes are a small portion of the class of phenylpropanoids characterized by a 1,2-diphenylethylene structure containing two phenyl radicals linked by a two-carbon methylene bridge. They can exist in two isomeric forms: *trans*-stilbene, which is not sterically hindered, and in the *cis*-stilbene, which is sterically hindered, and therefore less stable (Figure 1.23). Most stilbenes present in plants act as antifungal phytoalexins and have a low presence in the human diet. [58], [64], [65]

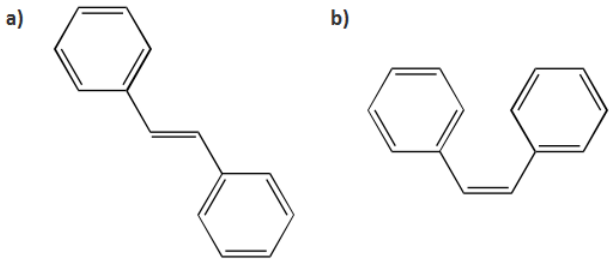

Figure 1.23 - The two isomeric forms of stilbene: a) trans-stilbene and b) cis-stilbene. Adapted from: [64]

As previously stated, the phenolic compounds are present in all plant foods and they are beneficial to health, due to their antioxidant activity, which makes them a natural source of antioxidants.

The phenolic compounds have antioxidant activity due to their ability to eliminate free radicals, donating hydrogen atoms or electrons, or chelate metal cations, and their structure is an important factor for these properties. [52]

Taking as example, the phenolic acids, their antioxidant activity depends on the number and position of hydroxyl groups relative to the carboxyl functional group. In this context, their antioxidant activity will increase with increasing degree of hydroxylation. The hydroxycinnamic acids have a higher antioxidant activity compared to the corresponding hydroxybenzoic acids, because the CH=CH–COOH group ensures greater H-donating ability and radical stabilization than in the case of the –COOH group. On the other hand, in the case of flavonoids the antioxidant activity is determined by changes in the rings B and C, Figure 1.19. The antioxidant activity is higher when there are double bonds in ring C and/or hydroxyl groups in rings B or C. [52]

The by-products of agro-industrial are rich in phenolic compounds and in recent years have attracted great interest as a source of natural antioxidants, such as rice hulls or by-products from olive oil production as olive pomace. [52]

The olives contain a number of phenolic compounds and their concentrations vary between 1-3% of the fresh pulp weight, having a great potential as an antioxidant and play an important role in the

organoleptic and nutritional chemical properties in olives, and consequently, in olive oil. On the other hand, residual products from olive oil extraction, such as the olive pomace, contain more than 90% of phenolic compounds present in fruits, for example, this residue is rich in hydroxytyrosol with a concentration ranging from 10 to 100 fold higher than in olive oil. This fact is due to their polarity, most phenolic compounds remain in the aqueous phase during the extraction of olive oil, but not all, given as they are also in part amphipathic. [43], [54]–[56]

The major classes of phenolic compounds present in olives are phenolic acids (which could be hydroxycinnamic acids, hydroxybenzoic acids as well as their derivatives), flavonoids and phenolic alcohols, which can be found in many fruits and vegetables. It may also be found phenolic compounds belonging to the class of secoiridoids, which are phenolic compounds that are only present in plants belonging to the *Oleaceae* family which includes *Olea europaea L.*, Table 1.11. [55]

Table 1.11 - Phenolic compounds can be found in olive. Adapted from: [43], [55]

Class	**Phenolic compound**
Hydroxycinnamic acids and derivates	Ferulic acid
	Caffeic acid
	p-Coumaric acid
	Verbascoside
Hydroxybenzoic acids and derivates	4-hydroxybenzoic acid
	3,4-dihydroxyphenylacetic acid
Secoiridoids	Oleuropein
	Demethyloleuropein
	Ligstroside
	Nüzhenide
Phenolic alcohols	Hydroxytyrosol
	Tyrosol
Flavonoids	Quercetin
	Rutoside or rutin
	Cynaroside
	Apigetrin
	Anthocyanins

Phenolic compounds classified as secoiridoids are characterized by having in its molecular structure the presence of any, elenolic acid[3] or its derivatives. They are produced from secondary metabolism of terpenes as precursors of several indole alkaloids and usually are derived from oleosides glucoside, which are characterized to be a combination of elenolic acid and a glycosidic residue. The oleuropein, dimethyloleuropein, ligstroside and nüzhenide are an examples of secoiridoids, and are the most abundant in olives. [55]

The oleuropein (Figure 1.24) is a glycosylated tyrosol ester of elenolic acid and is the result of the union of elenolic acid with hydroxytyrosol and glucose. It is responsible for the bitter taste of olives, and can constitute at least 14% of the dry weight of the olives. [43], [55]

The hydroxytyrosol (Figure 1.24) is a product of the hydrolysis of oleuropein. Their biological properties are similar to the oleuropein, such as antimicrobial, hypoglycaemic, hypolipidaemic, hypocholesterolic, antioxidant and free radical-scaveging actions, which means that this phenolic compound has aroused great interest. [43], [55]

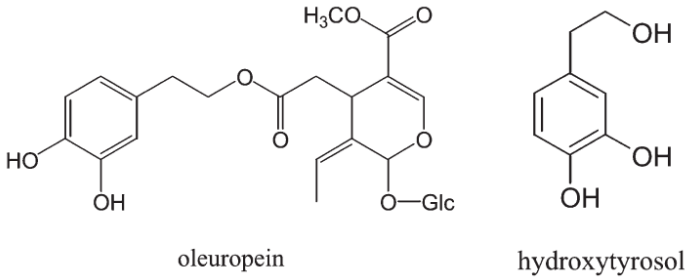

Figure 1.24 - Structures of Oleuropein and Hydroxytyrosol. Adapted from: [66]

Both oleuropein and hydroxytyrosol are naturally phenolic compounds found in olives, wherein the oleuropein is present in high amounts in the fruit of unprocessed olives, while hydroxytyrosol is more abundant in fruits and oil processed. [55]

The amount of phenolic compounds present in the olive fruit, olive oil or the by-products which are obtained in the extraction of olive oil, varies depending on multiple factors, such as, selection of olive, climate and geographical conditions, ripeness of the fruit, harvest time, soil composition; storage conditions before extraction and olive oil extraction method. These factors affect the amount of TPC, so also affect the composition of phenolic compounds. It should be noted that the constituent parts of olive also have a variation in the amount of TPC. [29], [43], [44], [55], [67]

[3] The elenolic acid is an exclusive compound present in olive oil and olive leaf extracts, and can be considered as a marker for maturation of olives. [109]

Table 1.12 - Total phenolic compound for different samples. TAE – tannic acid equivalent.

Samples		Content	Reference
Olive Oil		50 – 1200 (µg/g)	[68]
Olive Fruit	Pulp	13.9 – 30.5 (mg/g)	[55]
	Seeds	5.9 – 14.9 (mg/g)	
	Leaves (dried)	11.7 – 40.1 (mg/g)	
"*Alperujo*"		6.2 – 23.9 (mg/g)	[28]

In order to identify the phenolic compounds, it is necessary to isolate and recover them, and due to it, it's important to carefully execute the extraction stage, taking into account factors such as temperature, pressure or sample characteristics. Moreover, it is also important to consider the polyphenolic structure because these compounds may have several hydroxyl groups which may be conjugated to sugars, acids or alkyl groups. [50]

The method of liquid-liquid extraction (LLE) is a traditional method for extracting phenolic compounds in fruit and vegetables samples by using organic solvents such as methanol, ethanol or acetone, or in a mixture with water. [51]

The quantity and composition of phenolic compounds may vary in vegetable materials, moreover the phenolic compounds may interact with other compounds present in plants, such as carbohydrates and proteins, which can lead to the formation of complexes that can be very insoluble. So the solubility of phenolic compounds varies with the chemical nature of the plants, and vary from simple to very high polymerized. Furthermore, solubility may also be affected by solvent polarity chosen for the extraction.

The LLE extraction is usually carried out at high temperatures in order to minimize the duration of the process, since the phenolic compounds are degraded due to exposure to light together with the air, and temperature. [51]

So this method has disadvantages, such as the use of expensive and hazardous organic solvents, which are undesirable for health and disposal reasons, and also require a long time for analysis, giving rise to possible degradations of the phenolic compounds. [51]

To resolve the problems associated with liquid-liquid extraction, the solid-phase extraction emerged (SPE) although the cost of the equipment necessary for SPE is higher than for LLE. [51]

The solid phase extraction is based on differential migration processes during which the analytes are adsorbed on a solid adsorbent and then eluted with an organic solvent (e.g. ethyl acetate, methanol, acetonitrile or acetone) using cassettes, columns or syringes. The solvent to be used in the elution will be

chosen taking into account the type of adsorbent and the polarity of each analyte. Initially it was used adsorbents, of non-polar reversed-phase silica base, such as C_{18} adsorbent, for extraction of phenolic compounds from water samples. [69]

It has also been studied extraction of phenolic compounds from olive oil using normal phase SPE, because it was found that the C_{18} sorbent is inefficient to isolate polar components. [51]

Taking into account the problems associated with the LLE extraction, such as, the use and disposal of large and expensive quantities of organic solvents, and the cost of the equipment necessary for the SPE extraction is high. Therefore, it is necessary to replace these two methods by other methods which are more sensitive, selective, faster and environmentally friendly, for example, supercritical fluid extraction (SFE). [51]

SFE is a process for separating compounds from the matrices using supercritical fluids as an extraction solvent, such as CO_2. Supercritical fluids are characterized by having a combination of properties of gases and liquids: low viscosity, high diffusivity and good solvating ability. [51], [70], [71]

Supercritical fluid extraction (SFE) has many advantages over traditional methods, such as the use of low temperatures and reduced energy consumption and quality of the product, due to the absence of solute in the solvent phase. Another advantage is that the solvation power of a supercritical fluid can be adjusted by varying the temperature and pressure when it is near the critical point, which facilitates separation processes and solute recovery. The only downside to this method presents is to be limited to low or medium polarity compounds. [51], [72]

There is another more recent technique for the isolation of analytes present in solid samples such as phenolic compounds, which is the pressurized liquid extraction (PLE). This method uses organic solvents (e.g. methanol, acetone, or solvents mixed with water) at high pressure and temperatures above their normal boiling point. The technique consists of a solid sample was packed in stainless steel extraction cell and extracted with a suitable solvent at elevated temperatures (40-200 °C) and pressure (34-207 bar) for short periods of time (5-15 min) in that after the sample solution is flushed into a collecting flask with the aid of a compress gas. [51]

The PLE method has been shown to be very effective for extraction of phenolic compounds. [73]

Finally, there are the techniques of microwave-assisted extraction (MAE) and ultrasound-assisted extraction (UAE). [51]

The method MAE, is the application of microwaves in order to heat the solvent and the plant tissue and thus accelerate the extraction process. Compared to conventional extraction methods, the MAE has

a higher efficiency since the microwaves interact with polar molecules in the extraction media, the heat is generated and the internal pressure of the solid material is increased. [74], [75]

The great advantage of this method compared to methods SFE and PLE is that the extraction MAE can be performed in the absence of light, preventing degradation of phenolic compounds. [51]

In the case of UAE method, the potency of ultrasound provides a greater penetration of the solvent into cellular materials, in the case of extractive processes results in the rupture of the walls of the biological cells and facilitates the release of contents, which causes an improvement mass transfer. Consequently, improves significantly the kinetics of solvent extraction of organic compounds contained within the plants. In this method there is an increased efficiency and a reduction of the extraction time, due to cavitation[4] phenomena and mechanical mixing effect. [75], [76]

Since the UAE process is not a thermal process, the decomposition of the heat sensitive compounds is avoided. [75]

In both extractive processes (MAE and UAE) there are great advantages over the conventional extractive processes because there is a reduction in extraction time, greater efficiency, simplified manipulation and reduce solvent consumption. [77]

This work will perform the extraction of phenolic components using subcritical water, which has as main advantage the use of water as extracting solvent.

1.7 Sub-critical water

Sub-critical water (sub-CW), also identified as liquid hot water or hot-compressed water, is not a physically defined state, but a region below the critical point and above the triple point of water, Figure 1.25. The most practical definition is the hot water at temperatures ranging between 100 and 374 °C and maintained under high pressure to keep water in liquid state, i.e., kept at pressures above the vaporization curve and below the critical point, Figure 1.25. [78]–[80]

[4] "Cavitation is defined as the process of formation of the vapor phase of liquid when it is subjected to reduced pressures at constant ambient temperature. Thus, it is the process of boiling in a liquid as a result of pressure reduction rather than heat addition." [110]

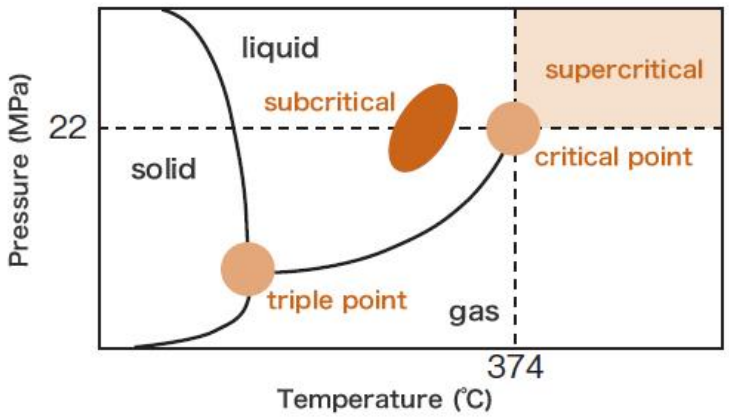

Figure 1.25 - Phase diagram of water. [81]

The sub-CW share the same advantages as the water at room temperature, because is environmentally innocuous, non-toxic, nonflammable, a safe solvent and is cheap compared with others organic solvents. Other advantages of using water is that the physicochemical properties can be altered by varying temperature and pressure and when the properties of water change the solubility of organics and inorganics compounds also changes. [79], [82], [83]

For sub-CW, the temperature is the most important factor, because in this case, the pressure will only ensure that the water is in the liquid state. The most important properties in sub-CW to be taken into account and those that vary with temperature are: the viscosity and surface tension, the dielectric constant and ionic strength.

When water is used as a solvent extraction, the dielectric constant is the most important factor, because it can be changed with temperature. At ambient temperature the dielectric constant is 80, but when the temperature increase to 250 °C (maintaining pressure in a way that the water remains in the liquid state), the dielectric constant is 27, which is almost identical to ethanol at ambient temperature, Figure 1.26. This can be explained by the fact, that when water is heated to high temperatures, the hydrogen bonds break causing the decrease of dielectric constant. Due to this fact, the water behaves as a less polar solvent, becoming a solvent more suitable for hydrophobic materials, such as organic compounds. For this reason, the sub-CW can be used in extraction processes, because with increasing temperature, the solubility of the ionic molecules decreases and consequently, the solubility of hydrophobic molecules increases. [78], [80], [84]

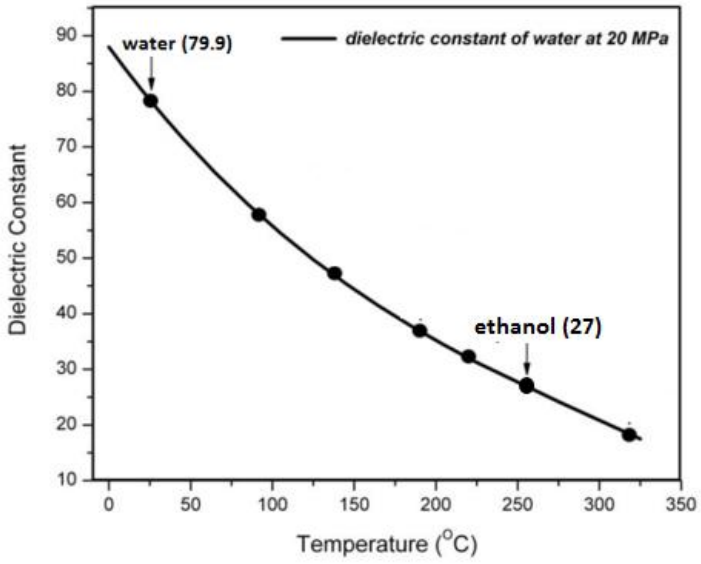

Figure 1.26 - Behavior of dielectric constant for water at different temperatures. Adapted from: [85]

For water the ion product is defined as $K_w = [H^+] [OH^-]$ concentration and its value increases with the increasing temperature, which can be observed in Figure 1.27. This occurs because the self-dissociation of water is endothermic. [84], [86]

At high pressures the ionic product of subcritical water can be some orders of magnitude higher than those of water in ambient conditions [86]. Because of that the water can play the role of an acid or base catalyst precursor, this can be explain for the higher concentration of H^+ and OH^- ions [86], in other words, the reaction equilibrium is shifted into the formation of H^+ or OH^- ions, but the pH does not suffer alterations.

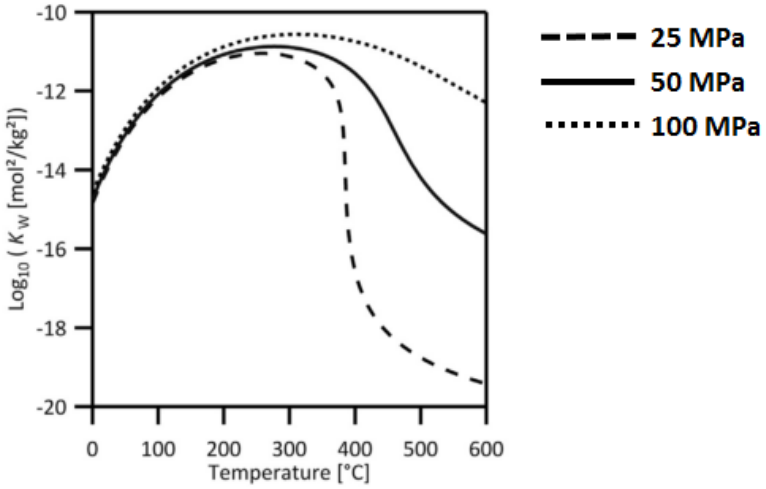

Figure 1.27 - Ionic product of water as a function of temperature. Adapted from: [82]

The viscosity of water varies with temperature and can be described by the Arrhenius-type equation, Equation 1.1, wherein μ is the dynamic viscosity (N.s/m^2) and T is the absolute temperature (K). [87]

$$\mu(T) = 2.414 \times 10^{-5} \times 10^{\frac{247.8}{(T-140)}}$$

Equation 1.1 - Viscosity of water as a function of its temperature. [87]

And as can be seen from Equation 1.1 and from Figure 1.28 , the viscosity decreases strongly with increasing temperature. [78], [87]

By increasing water temperature, the surface tension of water in contact with the air decreases, at 25 °C the surface tension of water is 72.0 mN/m (0.1 MPa) and 200 °C (1.5 MPa) decreases by about 50%, Figure 1.28. At the critical point the surface tension of water is zero. A lower surface tension of the extractant, which in this situation is water, leads to a better wetting of the sample, i.e. the area of contact between the extractant and the sample matrix increases, which leads to a more complete extraction. [87]

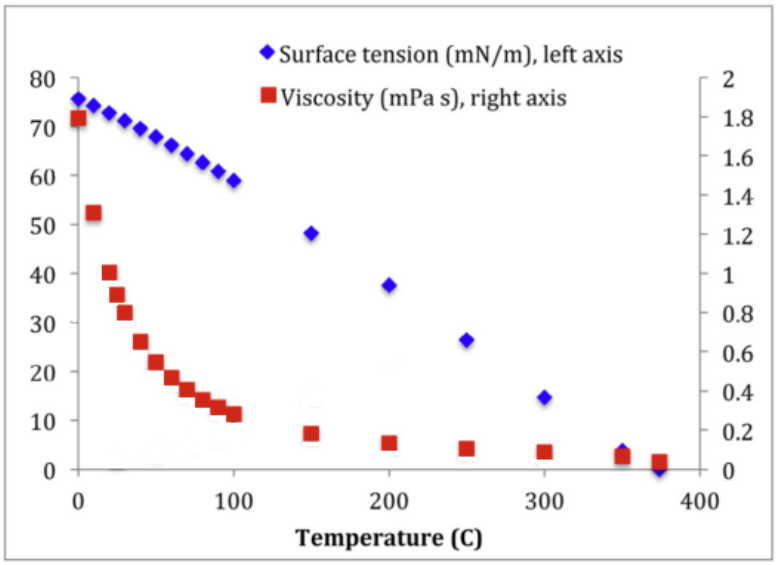

Figure 1.28 - Viscosity and surface tension of liquid water, at saturation pressure. Adapted from: [87]

Considering the aspects mentioned above, the subcritical water has a lower viscosity and surface tension than water at room temperature, which causes an increase in mass transfer rates from plant tissue matrix. [88]

The sub-CW has a wide range of applications, because have the properties that have been mentioned above. In this work, the sub-critical water is applied as solvent extraction of compounds with

added value present in agro-industrial residue, this is because, there has been increasing interest in ingredients of bioactive compounds from complex natural sources that can be used in food and pharmaceuticals products (e.g. antioxidants or anti-inflammatory compounds). This extraction method using sub-critical water can be used for analysis purposes or in industrial processes.

Before the extraction with sub-critical water is performed, it is necessary to take into account corrosion and its effects. Materials resistant to the effects of sub-CW are still limited, but many existing reactors consist of alloys such as stainless steel. Corrosion of these materials leads to the release of metal ions that are dissolved by sub-critical water and they may act as homogeneous catalysts for the proceeding reactions. [78]

Nowadays, there are studies which use the sub-critical water extraction to produce valuable materials, such as, wheat straw [89] and sugarcane bagasse [79] to produce reducing sugar, extraction of amino acids from soils [85] and extraction of antioxidants from grape pomace [88].

2 MATERIAL AND METHODS

2.1 Olive Pomace

In this work, the olive pomace used, was provided by a Portuguese producer of olive oil. The residue was lyophilized (lyophilizer CHRIST ALPHA 1-4, Braun Biotec International), it was first frozen to -20 °C in a flask, and then subjected to vacuum for about 1 week, depending on the amount of residue to lyophilize.

Due to the fact that the residue after drying does not provide a homogenization, containing large particles, olive stone fragments, which may lead to inconsistent results. A sieving was done to fractionate the residue into two samples, Gross Sample and Fine Sample, using steel sieves (Endecotts Laboratory Test Sieves) with pore diameters of 710, 1400, 2000 and 2360 µm. The fractions which were retained on the sieves of 2000 µm and 2360 µm were considered as *Fine Samples* (FS). Samples were retained on the screen 710 µm, 1400 µm and the base of column sieves were considered *Gross Sample* (GS). Note that the particles retained on the sieve of 1400 µm were further subjected to a previous grinding (IKA, Tube Mill Control).

Both samples, *Fine Sample* and *Gross Sample*, were characterized chemically. The *Fine Sample* was used to perform the extractions.

2.2 Extraction Methods

2.2.1 Lipids extraction

To determine the lipid content present in both samples a *Soxhlet* extraction was performed. 2 g of sample were inserted on a filter paper package, which was placed into a *Soxhlet* apparatus for 4 hours using 65 mL of n-hexane (Carlo Erba Reagents). After extraction the package was placed overnight in an oven (WTC Binder) at 40 °C to remove the solvent, after this procedure the dried residue was weighted. The obtained solution was placed in a tared flask and subjected to nitrogen, in order to remove the solvent by evaporation, only remaining oil in the bottle, which was then weighted.

2.2.2 Carbohydrates extraction

The procedure for extracting the fraction of carbohydrates present in the dry alperujo, allows to separate the carbohydrates into soluble sugars (monosaccharides and oligosaccharides that are soluble in mixtures of water/alcohol are all considered) and insoluble sugars (which are all complex carbohydrates, such as cellulose). Lipids and lignin are also separated by this procedure.

The procedure begins by performing an extraction of the lipids in the sample, following the procedure described in the Chapter 2.2.1, followed by a hydro-alcoholic extraction, to extract the soluble sugars, and finally an acid hydrolysis, to extract the insoluble sugars.

Lignin is determined by taking into account the weight of the protein and ash resulting dry residue of acid hydrolysis. The procedures for the quantification of protein and ash are described in Chapters 2.3.1 and 2.3.2, respectively.

Hydro-alcoholic extraction: based on the article by Deng *et al.* (2011) [90], 0.8 g of the resulting residue from the *Soxhlet* extraction was extracted with 40 mL of ethanol: water (80:20 v/v) in an ultrasonic bath for 15 minutes at room temperature. After that the solution was centrifuged (Beckman Coulter Avanti Centrifuge J-26 XP) at 10 000 rpm for 10 min at 4 °C. The process was repeated three times. The resulting three supernatants were combined and the ethanol was evaporated in a rotary evaporator (Nahita), at 50 °C under vacuum. At the end was added 100 mL of milli-Q water to the remaining solution. Of the obtained solution was removed (5 mL) for a vial which was placed to dry in an oven at 100 °C for 24 h. The remaining solution was used to perform the analysis of carbohydrates, Chapter 2.3.4.

The residue resulting from the centrifugation, after extracting the lipids and soluble sugars, was placed in an oven (WTC Binder) to dry at 40 °C overnight.

Acid hydrolysis extraction: based on a protocol by Sluiter *et al.* (2008) [91], in a Schott flask was placed 0.3 g of dry residue remaining from the hydro-alcoholic extraction and was added 3 mL of H_2SO_4 72% (w/w). The mixture was put to incubate in a 30°C water bath for 1 hour. To dissolve the residue, the mixture was placed under magnetic stirring (150 rpm). After this 84 ml of milli-Q water were added to dilute the mixture to 4%. The mixture was placed to incubate in a silicone (Baysilone M350) bath at 121 °C under magnetic stirring (150 rpm) for 1 hour. The cooled mixture was filtered using a funnel or crucible with sintered disc, previously weighted. The remaining residue was washed with distilled water and dried

in an oven (Cassel) at 105 °C overnight, after drying, the solid was weighted. The supernatant was then analyzed to quantify carbohydrates, Chapter 2.3.4.

2.2.3 Phenolic compounds extraction

To extract the phenolic compounds, present in the "*alperujo"*, a hydro-alcoholic extraction was performed, based on procedures used by Silva et al (2015) [92]. To 1 g sample of dry olive pomace was added 20 mL of ethanol: water solution (25:75 v/v). The mixture was placed to incubate in a water bath at 50 °C for 18 hours under constant magnetic stirring (150 rpm). The mixture was then filtered and the obtained solution was used for quantification of phenolic compounds by following the procedure described in Chapter 2.3.5.

2.3 Analytical Methods

2.3.1 Protein quantification

The quantification of protein was based on the protocol by Hames *et al.* (2008) [93]. An *eppendorf* was placed about 2-3 mg of dry sample of olive pomace, and this was subjected to the elemental analysis - CHNS performed by Laboratório de Análises REQUIMTE - LAQV in the Chemistry Department of the FCT/UNL, to determine the nitrogen content. The protein content was determined taking into account the nitrogen content, using a conversion factor of 6,25.

2.3.2 Ash quantification

The determination of the ash content in the residue was based on the methodology by Sluiter *et al.* (2005) [94], in a porcelain crucible was weighed about 0.2-0.8 g dry residue and was placed in a muffle at 550 °C for 4 hours. At the end of this time the crucible was placed in a desiccator to cool down and was then weighted. The ash content was determined by mass difference.

2.3.3 Water content quantification

The water contents were determined taking into account the protocol described by Sluiter *et al.* (2008) [95]. The residue was placed in a petri dish and placed in an oven at 105 °C for 4 hours. At the end of this time, the sample was allowed to cool and weighted. By the mass difference was determined the water content.

2.3.4 Total carbohydrates quantification

The quantification of total carbon hydrates in a given sample was performed by a colorimetric method, the phenol-sulfuric method, based on a modification of the method described by Masuko *et al.* (2005). [96]

The quantification is made by using a calibration curve constructed with solutions of D(+)-glucose monohydrate, SIGMA Aldrich. The solutions were prepared from a stock solution with a concentration of 1 g/L (in a volumetric flask of 100 mL was added 0.1 g of D(+)-monohydrate glucose (SIGMA Aldrich) and was added to milli-Q water until it reaches the volume) with concentrations of 0.005; 0.01; 0.025; 0.05 to 0.1 g/L. The blank was milli-Q water.

In a test tube, was added 500 µL of solution rich in sugar, and added 1.5 mL of H_2SO_4 (Panreac 96%) and 300 mL of a 5% (w/v) aqueous solution of phenol (Sigma Aldrich 99-100%). The mixture was stirred using a vortex (Vortex Mixer, Labnet) and was incubated in a dry bath (Accu BlockTM Digital Dry Bath) at 90 °C for 5 min. At the end of this time, the mixture was again stirred and cooled in a water bath at room temperature. The absorbance of the mixture was measured at 490 nm in a spectrophotometer (DU800 Spectrophotometer from Beckman Coulter, Brea, USA). The sugar concentrations are expressed in g/L glucose equivalent.

2.3.5 Total phenolic content quantification

The method to quantify the content of phenolic compounds is divided in two 2 steps: the Protein Precipitation method, based on the procedure described by Sivaraman *et al.* (1997) [97] to remove protein

present in the sample, and the *Folin-Ciocalteu* method, based on the procedure described by Waterhouse (2001) [98], a colorimetric method for counting the phenolic compounds present in the sample.

Method for precipitation of protein, based on the procedure described by Sivaraman et al. (1997) to remove protein present in the sample and the Folin-Ciocalteu method based on the procedure described by Waterhouse (2001), a colorimetric method for counting the phenolic compounds present in the sample.

The quantification is made using a calibration curve constructed from gallic acid monohydrate (SIGMA – Aldrich) solutions. The solutions were prepared from a stock solution with a concentration of 5 g/L (in a volumetric 100 mL flask was weighted 0.5 g of gallic acid monohydrate (SIGMA – Aldrich) and was added 10 mL of ethanol (Carlo Erba Reagents, 99.9%) and milli-Q water until the volume balloon) with concentrations of 25, 50, 100, 150, 250 and 500 mg/L. The blank was milli-Q water.

Protein Precipitation method: in a eppendorf, to 800 µL of sample was added 120 µL of 100% (w/v) trichloroacetic acid, TCA (Scharlau 99.5%). The mixture was stirred using vortex (Vortex Mixer, Labnet) and stored at -20 °C for 5 min, then at 4 °C for 15 min. After that time, the mixture was centrifuged (Biofuge 13, Heraeus Sepatech) for 15 min at 12000 rpm. The precipitate was discarded.

Folin-Ciocalteu method: in a test tube was added 20 µL of the recovered supernatant/standard solutions of gallic acid/blank solution, 1.58 mL of milli-Q water and 100 µL of Folin-Ciocalteu reagent (MERK). The mixture was stirred using vortex and incubated for 1 to 8 min at room temperature. Then it was added 300 µL of sodium carbonate (Sigma) solution and the mixture was again stirred with the help of vortex. The mixture was then incubated in a dry bath (Accu BlockTM Digital Dry Bath) at 40 °C for 30 min. Finally, the absorbance was measured at 750 nm in the spectrophotometer, DU®800 Spectrophotometer from Beckman Coulter, Brea, USA. The sample concentration was determined using gallic acid standard curve and expressed in mg/L GAE (gallic acid equivalent).

2.3.6 HPLC methods for characterization and quantification

A. HPLC method for carbohydrates analysis

The HPLC method was used to identify and quantify the carbohydrates: xylose, mannose, fructose, arabinose, galactose, glucose and sucrose. This analysis was performed by Laboratório de Análises REQUIMTE - LAQV, in the Chemistry Department of the FCT/UNL, using the Dionex ICS-3000 system with electrochemical detection (ED) and Photodiode Array (PAD). Were used the 4 x 50 mm Dionex BorateTrap and 4 x 50 mm Thermo BioLC Dionex AminoTrap, as pre-columns.

In the first method was used the 4 x 250 mm Thermo Dionex CarboPac PA10 column, at a constant temperature of 30 °C. As mobile phase was used a solution of NaOH at 18 mM at a constant flow rate of 1 mL/min. For the second method was used the 4 x 250 mm Thermo Dionex CarboPac SA10 column, at a constant temperature of 40 °C. A solution of NaOH 1 mM was used as mobile phase, at a constant flow rate of 1.2 mL/min. In both methods, the injection volume was 25 µL.

For analysis was used patterns of various sugar monomers previously prepared.

B. HPLC method for phenolic compounds analysis

The HPLC method was used to identify and quantify the phenolic compounds: hydroxytyrosol, tyrosol, oleuropein, quercetin, caffeic acid, ferulic acid and p-coumaric acid. This analysis was performed by Laboratório de Análises REQUIMTE - LAQV, in the Chemistry Department of the FCT/UNL, using the Dionex ICS-3000 system with Photodiode Array (PAD). The absorbance is measured using a wavelength of 280 nm with a UV / Vis detector. For the analysis was used a reverse-phase polymeric C_{18} column, Novapak C_{18} Waters 150 x 4 mm. The mobile phase is a mixture of two eluents, A and B. Eluent A is a solution with 10% methanol and 2% acetic acid, and eluent B is a solution with 90% methanol and 2% acetic acid. This method has an injection volume of 25 µL and flow rate of 0.5 mL/min. the column was kept at a constant temperature of 25 °C.

2.3.7 DPPH assay – Antioxidant activity

The DPPH assay, is a discoloration assay, whose purpose is, evaluate the antioxidant activity of a sample, in this case the olive pomace extracts. The DPPH radical, is a stable organic nitrogen centered free radical, with a dark purple color, when it is reduced to a non-radical form due to the presence of

antioxidants becomes colorless or even change to a yellow color. This method is based on measurement of loss of DPPH color at 517 nm, in a spectrophotometer, after reaction with solutions prepared from the olive pomace extract. [99]

For performing this method, it was necessary to prepare three solutions: the DPPH solution, which was used to prepare the stock solution; the extract solution, that was used to prepare the dilutions required by assay, and the stock solution, used in DPPH assay.

DPPH solution: in a volumetric flask of 100 mL was dissolved 24 mg of DPPH (Aldrich Chemistry) in 100 ml of methanol. This mixture was then stored at -20 °C for a minimum of 2 hours.

Extract solution: the olive pomace extract was dissolved, which is the freeze-dried extract that resulted from hydrolysis of olive pomace, in the solution water:methanol (50:50, v/v). The volume of water:methanol (50:50, v/v) and the amount of freeze-dried extract depends on the volume and concentration of the extract solution, in this work the concentration of the extract solution was 5000 mg/L.

The dilutions of extract solution were prepared for a range of concentrations between zero (blank solution, only water:methanol solution) and 4000 mg/L.

Stock solution: 20 mL of the DPPH solution was added 90 mL of methanol (SIGMA – Aldrich, 99.8%) in an amber flask. The solution was stirred and the absorbance was measured at 517 nm in a spectrophotometer (DU®800 Spectrophotometer from Beckman Coulter, Brea, USA). If the absorbance was above 1.1, it would be necessary to add additional methanol, starting with 5 mL and gradually increasing until absorbance measure was close to 1, in other words, the absorbance of the stock solution had to be between 1 and 1.1.

DPPH assay: in amber vials were added 4 ml of stock solution and 150 µL of each dilution of the extract solution. The blank was prepared by adding in an amber flask, 4 mL of the stock solution and 150 µL of water: methanol solution (50:50, v/v). At the end of that time, the samples were mixed well and the absorbance was measured at 517 nm. To evaluate the antioxidant activity of olive pomace extracts, the half maximum effective concentration (EC_{50}) was calculated from the inhibition curves obtained. To evaluate the inhibition of the free radical by each sample, the following equation was used, where $DPPH_{blank}$ is the absorbance of the blank and $DPPH_{extract}$ is the absorbance of the sample with extract:

$$\%\ of\ inhibtion = \frac{DPPH_{blank} - DPPH_{extract}}{DPPH_{blank}}$$

2.4 Sub-CW Extraction Apparatus and Methodology

It was used the apparatus shown in Figure 2.1. to make the extraction of compounds with additional value from olive pulp using subcritical water as extraction solvent.

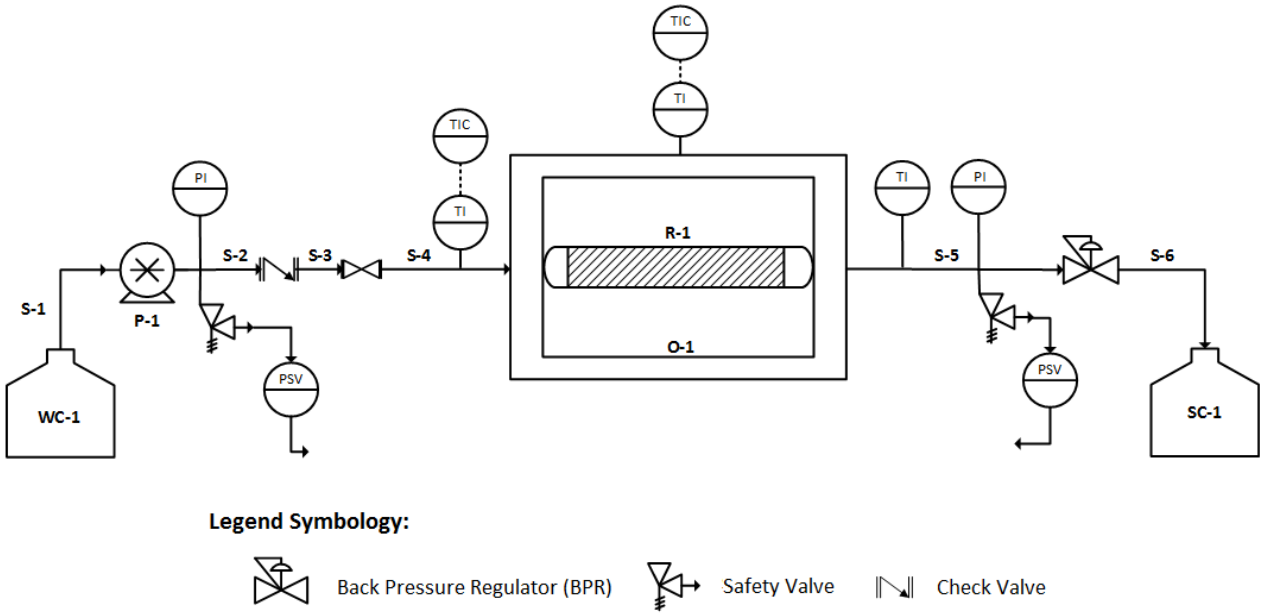

Figure 2.1 - Schematic of the sub-CW extraction apparatus.

Legend: WC– Water container; P– Pump; R – Reactor; O – Oven; SC – Sample container; S – Stream
PI – Pressure indicator; TI – Temperature indicator; TIC – Indicator and controller temperature; PSV – Pressure safety valve.

The installation consists of a pump (Preparative Pump 1800, KNAUER) that is connected to a container of distilled water, which pumps the water through a high pressure pipe connected to the reactor. The water before entering into the reactor, is heated by a heating wire which is connected to a temperature controller, the input pressure is indicated by pressure indicator. The reactor is filled with the dry olive pomace and glass beads, at the exit of the reactor was placed a porous disc. The body of the reactor is stainless steel and has a 5 cm external diameter, 51 cm length and 2.6 cm internal diameter. The output of the reactor also has a temperature and pressure indicator. The pressure in the system is regulated by a Back Pressure Regulator, BPR (Tescom Europe®, 26-1000). The output current is collected for analysis. In the installation, all valves and fittings used are from HIP and SWAGELOK.

In all tests the pressure was kept constant at 60 bar and experiments were performed for three different temperatures: 140, 170 and 200 °C. The water flow was also kept constant at 10 mL/min in all assays.

To perform an experiment, the pump is turned on with the flow rate selected and is checked if there is leaks in the apparatus. If there is no leak, it begins the pressurization of the system. When the pressure reaches 60 bar, is checked again if there is leak in the apparatus, if there is no leak, the water heating wire and the oven are turned on, and sample collection starts.

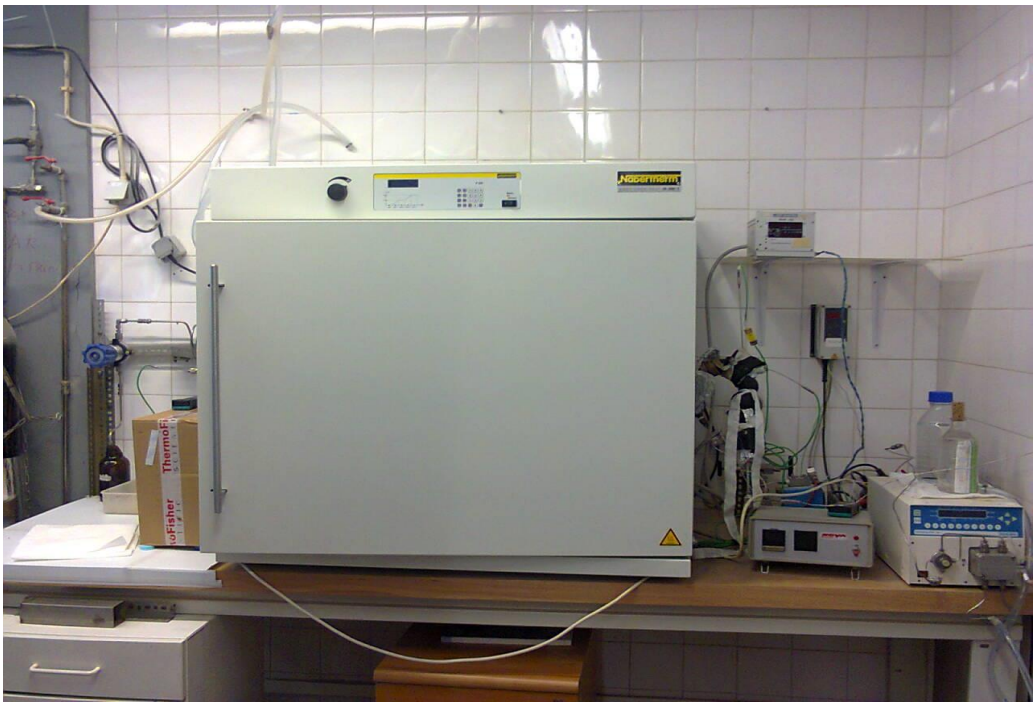

Figure 2.2 - Sub-CW apparatus used for extraction and hydrolysis of dried sample from "alperujo".

In the assay, the sample collection is made according to Table 2.1 and are used Schott flasks, to collect the accumulated, or Falcon tube (50 mL), for the collection of fractionated.

When the assay temperature is reached and kept constant, the sample collection is performed for another 15 minutes, and the samples are collected in Falcon tubes every 5 min.

All the collected samples are stored at 4 °C. For each sample, is made the total carbohydrates and total phenolic compounds quantification, following the protocols described in Chapter 2.3.4 and Chapter 2.3.5, respectively. 10 mL of each sample are lyophilized, to obtain what will henceforth be called olive pomace extract, which will be used in the assay DPPH in the preparation of the extract solution, Chapter

2.3.7, and for the quantification of protein, Chapter 2.3.1. 2 mL of each sample, 1 mL without enzyme and 1 mL of sample with enzyme *ViscozymeL* (SIGMA), are used for quantification and identification of carbohydrate based on HPLC analysis, Chapter 2.3.6A. And 1 mL for quantification and identification of phenolic compounds based on HPLC analysis, Chapter 2.3.6B.

Table 2.1 - Scheme for the collection of samples.

Range temperature (°C)	**Assay temperature (°C)**		
	140	170	200
[0-140]	The sample collection is made of 5 in 5 minutes, in a Falcon tube	The sample collection is made by accumulation, in a Schott flask	The sample collection is made by accumulation, in a Schott flask
[140-170]		The sample collection is made of 5 in 5 minutes, in a Falcon tube	The sample collection is made by accumulation, in a Schott flask
[170-200]			The sample collection is made of 5 in 5 minutes, in a Falcon tube

To the residue, result of each assay, is performed: lipid extraction (Chapter 2.2.1), extraction of the insoluble carbohydrates (Chapter 2.2.2) and respective quantitation following the protocol described by Chapter 2.3.4. It also performed the protein and ash quantification, and the DPPH assay, following the protocol described by Chapters 2.3.1; 2.3.2 and 2.3.7, respectively.

2.5 Stability assay

The purpose of stability assay, is to see the evolution of the amount of total phenolic compounds and antioxidant activity over time, taking into account that the phenolic compounds degrade with temperature and when subjected to light, reducing antioxidant activity.

Over time (days) the total phenolic compounds were quantified using the protocol described in Chapter 2.3.5, whilst the DPPH assay followed the protocol described in Chapter 2.3.7.

All samples involved in the stability assay, are the result of accumulation of the samples obtained from the assay at 140 °C. In the stability test were prepared, the following samples:

- CT sample (sample stored at **C**old **T**emperatures) was stored at 4 °C in a Schott flask, covered in aluminum foil;
- RT sample (sample stored at **R**oom **T**emperatures) was subjected to nitrogen and stored in a falcon tube at room temperature and unprotected from light;
- FRT sample (sample stored in the **F**reezer and at **R**oom **T**emperature);
- FT sample (sample stored in the **F**reeze **T**emperature)

The CT sample and RT sample samples were compared in order to study the evolution of the concentration of phenolic compounds and evaluate the antioxidant activity over time. For this comparison a reference analysis was performed days after the test at 140 °C for this analysis was used, the total sample of the test (this sample results from the accumulation of rich liquor in sugars obtained by the assay at 140 °C) the results of this analysis are from now on designated as zero. For CT and RT samples the quantification of the phenolic compounds was made 20, 30 and 60 days after being determined the reference data. Also, the antioxidant activity was evaluated for the same days after having performed the reference analysis, following the DPPH assay protocol, described in Chapter 2.3.7.

The FRT (sample stored in the **F**reezer and at **R**oom **T**emperature) and the FT (sample stored in the **F**reezer **T**emperature samples were both analyzed (quantification of phenolic compounds and evaluate the antioxidant activity) 68 days after the extraction, with the purpose of being compared with CT and RT samples, to check if there is change in the antioxidant activity and in the total phenolic compounds for different storage methods.

3 RESULTS AND DISCUSSIONS

3.1 Composition of "*alperujo*"

The first step of the work, had as objective drying the biomass, which in this study was an agro-industrial residue from the olive oil extraction, "*alperujo*" or better known as olive pomace. The drying of the residue was performed by lyophilization, having verified that the olive pomace has a water content of 71.3% +/- 0.30%, consistent with the paper by *Alburquerque* et al. (2003). [28]

After the water was removed, the "*alperujo*" was divided into two samples, Fine Sample (FS) and Gross Sample (GS), depending on the size of the particle. The FS sample comprised particles with sizes below the 1400 µm and, the GS sample would be the particles with diameter between 1400 and 2360 µm.

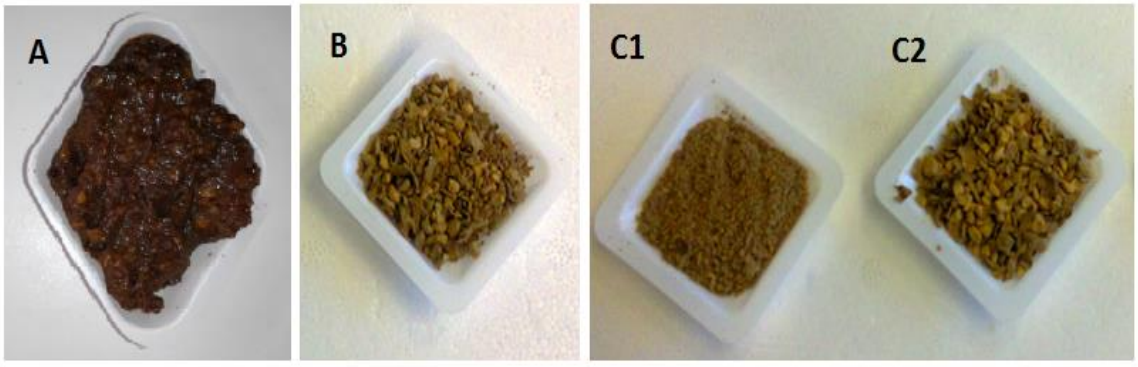

Figure 3.1 - A - Olive pomace; B - Dried olive pomace; C1 - Fine Sample (FS); C2 - Gross Sample (GS).

The composition was determined for both samples, using the methodologies described in Chapter 2.2 and Chapter 2.3. The major components identified in both samples, FS and GS samples, were proteins, lipids, ash, carbohydrates and lignin, Table 3.1.

The paper that was used as a reference for the results of the composition, of *"alperujo"* was the paper by Alburquerque *et al.* (2003) [28], but it should be noted the chemical characterization of "*alperujo*", in the paper, was made from the non-homogenized residue, i.e., there was no separation of the residue in terms of particle size, as was done in this work.

For both samples the results of soluble sugars and ash content are within the ranges set in the literature. [28]

Table 3.1 - Composition of the dried olive pomace, in wt.%. Prefixes: FS = Fine Sample; GS = Gross Sample.

Components	FS (wt. %)	GS (wt. %)
Lipids	55.5 ± 2.36	13.5 ± 0.07
Water soluble carbohydrates	4.9 ± 0.48	1.7 ± 0.35
Water insoluble carbohydrates	13.2 ± 1.22	22.7 ± 1.27
Lignin	7.8 ± 0.63	59.1 ± 1.63
Protein	10.5 ± 0.01	1.3 ± 0.06
Ash	8.0 ± 0.01	1.4 ± 0.03

In the case of lipids it can be seen that the results obtained for the FS and GS samples, are within the range defined in the paper by Alburquerque *et al*. (2003) [28], but the percentage of lipids in the FS sample is higher compared to the GS sample, which was expected, because the GS sample is constituted mainly by olives pit residues (stone wall and seed). In the Figure 3.2, can be observed the color differences between the two samples, the FS sample present an oil with a green color a while the color obtained from GS sample is more clear color.

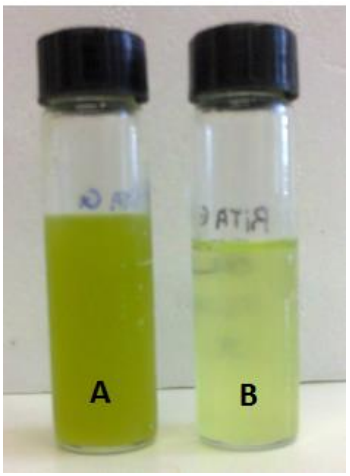

Figure 3.2 - Oil obtained from the Soxhlet extraction. A - Fine Sample (FS); B - Gross Sample (GS).

The results for the lignin, in the case of the FS sample, and the protein, in the GS sample, were unexpectedly low, they are not within the limits set in the literature. Moreover, the consistency regarding the insoluble carbohydrate, for both samples was low. In the work by Nogueira *et al.* (2015) the carbohydrates presents in "*alperujo*" are: glucose, xylose, mannose, fructose, arabinose and galactose. The main monosaccharide in the soluble sugar fraction is glucose (75%). On the other hand, xylose and glucose are the most likely to exist in the non-soluble sugar fraction (46% and 31%, respectively). [48]

To perform the quantification and identification of phenolic compounds present in both samples from the "*alperujo*" it was necessary to perform an extraction of these compounds, using the hydro-alcoholic extraction method, following the methodology described in Chapter 2.2.3. For the quantification of these compounds, the colorimetric method was used, following the Folin-Ciocalteu method, described in the Chapter 2.3.5. For both samples was performed the identification and quantification of the phenolic compounds by HPLC following the methodology described in Chapter 2.3.6B.

As mentioned in Chapter 1.6, the variety of olive, the fruit ripening, harvest time, the storage conditions before the olive oil extraction, and the selected extraction method, caused variations in the TPC content present in the olive fruit and in sub-products that are obtained in olive oil extraction. The composition of the soil and climatic and geographical conditions are also factors that influence the TPC content. For both sample the concentration of phenolic compounds obtained was less than 1% of the dry weight, not being a significant contributor to the chemical composition and were therefore left out of the table.

The results of TPC, obtained for both the samples are low, compared to the results obtained by Albuquerque *et al.* (2003) [28], about 6.2 to 23.9 mg water-soluble phenols/g dry weight, but the FS sample has a high content of TPC in relation to the GS sample, Table 3.2. A possible explanation for the difference content in phenolic compounds present in the FS and GS samples, in comparison to the literature, is the storage conditions of "alperujo". Alperujo was stored at room temperature for more than a year, which may have led to the proliferation of fungi.

Table 3.2 - Phenolic compounds quantification.

Content (mg/g dried sample)	**Fine Sample (FS)**	**Gross Sample (GS)**
TPC	10.9 ± 0.59	4.1 ± 0.42
Hydroxytyrosol	0.1 ± 0.01	0.9 ± 0.03
Tyrosol	0.7 ± 0.01	0.5 ± 0.01
Oleuropein	n.d	0.03 ± 0.01

n.d- not detected

As previously stated, the TPC content in the alperujo can be affected by factors such as the variety of olives fruit or the processing techniques used for oil extraction, so that these factors will also affect the composition of phenolic compounds.

The oleuropein and hydroxytyrosol are phenolic compounds found exclusively in olive, according to the paper by Silva *et al.* (2005), the oleuropein is found in high amounts in olive fruit unprocessed, while the hydroxytyrosol is more abundant in olive fruit processed an in olive oil. [55]

One reason, according to the paper by Lafka *et al.* (2011), for oleuropein is detected in small amounts in the by-products of olive oil extraction, such as the "*alperujo*". Its concentration decreases by enzymatic degradation and the concentration of its free components increases during the stage of maturation and processing. [56]

Another reason for the TCP content and the low quantity of phenolic compounds, is present during the extraction of olive oil preparations are added commercial enzymes in order to increase the yield and quality of the oil, or to improve the release of phenolic compounds. The enzymes most commonly used are *Olivex*[5] and *Cytolase 0.*[6], which are complex preparations of fungal origin rich in pectinolytic activities with hemicellulolytic and cellulolytic side activities. [100]

As can be observed from Table 3.2 hydroxytyrosol and tyrosol are the compounds with higher concentration, as expected, since in the literature these phenolic compounds are the major compounds of the "*alperujo*". It was also observed that the concentration of oleuropein for both samples is low, which is explained by the fact that oleuropein is present in larger quantities in unprocessed olive.

It can also be noted that the TPC contents for both samples are high, compared to results obtained by HPLC, whereby it may suggest that there are other phenolic compounds, which have not been properly identified due to the fact that the HPLC peaks for the compounds in question do not match any standards.

Eventually, for the identification of these compounds an HPLC-MS analysis could be performed to compare the masses of these possible compounds with masses that exist in the literature.

The compounds not detected may be *p*-coumaric acid, ferulic acid, or degradation products of hydroxytyrosol or tyrosol. Observing the GS sample chromatogram, Appendix 6.3 - Figure 6.1, it can be said that these compounds are more polar than the oleuropein.

[5] The enzyme *Olivex* is a pectinase preparation with low levels of cellulase and hemicellulase from *Aspergillus aculeatus*. [111]

[6] The *Cytolase 0.* is an enzyme preparation containing pectinase, cellulolytic and hemicellulolytic enzymes and some minor enzymes. [112]

3.2 Sub-CW Extraction

3.2.1 Process efficiency

In this work the influence of temperature on the extraction efficiency of sub-critical water (sub-CW) for "*alperujo*" was studied.

It was observed a change in the color of the samples recovered during the extraction process, for all tests. At first, the collected sample showed a clear brown color and as the continued extraction, with the gradual increase in temperature until it reaches its maximum value (200 °C) the color of the sample was more intense, showing some turbidity.

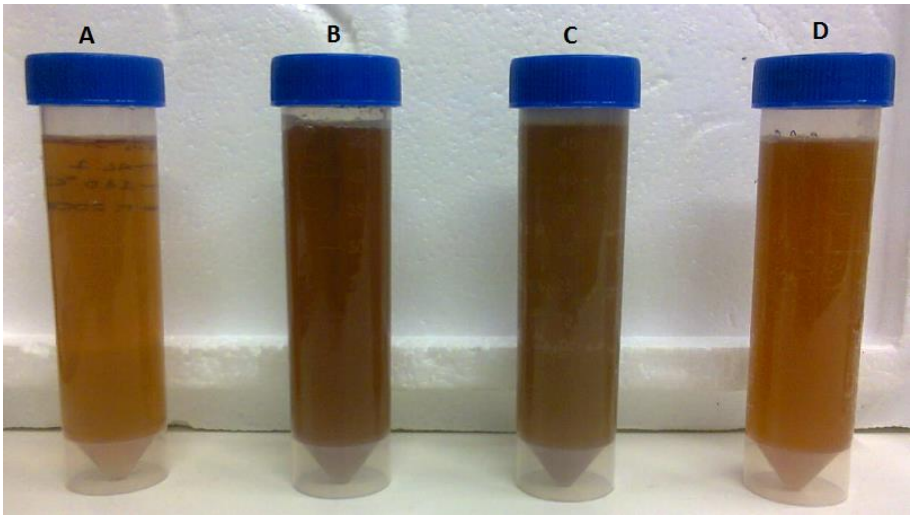

Figure 3.3 - Samples collected during the extraction test at 200 °C, in the temperature range (P = 60 bar; Flow rate = 10 mL/min): A – [0-140] °C; B – [140-170] °C; C – [170-200] °C; D – step of 200 °C.

To evaluate the influence of temperature on the extraction of Fine Sample (FS) of the "*alperujo*", the assay was performed reaching a maximum temperature of 140, 170 and 200 °C, maintaining the same initial amount of biomass and constant flow.

The biomass conversion is given by the Equation 3.1, wherein $FS_{inicial}$ is the amount of Fine Sample placed in the reactor (g) and FS_{final} is the amount of Fine Sample that is recovered from the reactor at the end of the assay (g).

$$Conversion\ (\%) = \frac{FS_{inicial} - FS_{final}}{FS_{inicial}} * 100$$

Equation 3.1 -Biomass conversion.

The yield of water-soluble compounds (WSC), is calculated by taking into account the amount of extract obtained, Equation 3.2, and this extract is obtained by lyophilization of the samples collected during the assay.

$$Yiel\ of\ WSC\ (\%) = \frac{\frac{A_{lyophilized} * V_{sample}}{V_{lyophilized}}}{FS_{inicial}} * 100$$

Equation 3.2 - Calculation of the yield of water-soluble compounds.

where, $A_{lyophilized}$ is the amount of lyophilized sample (g), V_{sample} is the volume of sample collected during the assay (mL), $V_{lyophilized}$ is the volume of sample it was lyophilized (mL).

As expected, biomass conversion and yield of WSC, increases with the increase in temperature, Table 3.3. Because with the increase in temperature, the ionic product of water increases, making the water a stronger catalyst for the hydrolysis of biomass. Another reason is the decrease of water viscosity with the increase in temperature, leading to an increase of mass transfer in matrices of plant tissues. [78]

As can be seen from Table 3.3, the results of both the conversion and yield are low. One explanation is the small amount of biomass used in the assays. The temperature may also explain the differences in the yields obtained, where the best yield of water-soluble compounds was achieved at 200 °C (30%), around 25% higher than at 140 °C.

The heating time and time duration of the assay, are factors which also influence the yield of soluble compounds and in turn the conversion. The higher was the target assay temperature, higher was the duration of the assay, which leads to a higher amount of extract recovered.

Table 3.3 - Yield of water soluble compounds (WSC) and conversion FS for different assay conditions. P = 60 bar.

Assay conditions			**(%)**	
Temperature (°C)	Flow rate (mL/min)	Amount of biomass (g)	Yield	Conversion
140	10	31.85	5	19
170		31.86	9	22
200		25.68	30	43

It can also be noted, that the quantity of biomass which disappears during the experiment, about 14% of the mass it is not recovered from the reactor. A possible explanation, is the cooling method of the

system, this cooling is done by increasing the water flow in order to reduce the cooling time. During the cooling phase, which may last for about 1 hour, some compounds can be extracted, and they are not considered in the quantification of the water-soluble compounds. The conversion increase from around 19% at 140 °C to around 43% at 200 °C.

In overview, the differences in yields, are due to:

- The temperature, which influences the ion product and the viscosity of the water, increasing the mass transfer;
- The target temperature assay, the higher the target temperature, higher is the heating time, and therefore higher duration of the process, and hence, more compounds are extracted;
- The amount of biomass placed in the reactor, more biomass in the reactor, more soluble compounds can be recovered.

3.2.2 Phenolic Content

One of the main objectives of this study, was to evaluate the extraction of phenolic compounds from "*alperujo*", in this case the Fine Sample (FS), performing extractions with subcritical water at different temperatures, Chapter 2.4. Because as noted in Chapter 1.6, the olives and by-products of olive oil extraction, such as the "*alperujo*", contain several phenolic compounds, having a great potential as an antioxidant. The oleuropein and its derivatives, tyrosol and hydroxytyrosol, are the most common phenolic compounds in olive pomace, wherein hydroxytyrosol is the phenolic compound found at higher concentration. These compounds have great benefits for health and can prevent many diseases such as atherosclerosis or heart disease, for this reason has been growing the interest in this type of compounds.

The extraction of phenolic compounds from biomass is influenced by several factors, such as, the method and solvent of extraction selected, the extraction conditions (extraction time, temperature and flow rate of the solvent), the sample (type of sample, particle size, time and conditions of storage); solid-liquid ratio; presence of interfering substances, etc. [101]

Different extracting solvents influenced the TPC concentrations, which can be explained as a result of polarity if the solvent and thus different extractability among the phenolic compounds. [102]

During the sub-CW extraction of polyphenols from plant materials, diverse phenomena occur including thermal degradation, and selective polyphenol extraction, all of which are highly dependent on extraction temperature and duration. [88]

The TPC quantification was performed following the Folin-Ciocalteau method described in Chapter 2.3.5. It was also performed the identification and quantification of the phenolic compounds by HPLC following the method described in Chapter 2.3.6B.

Table 3.4 - TPC and quantification of the major phenolic compounds present in the FS sample to different conditions. P = 60 bar; Flow rate =10 mL/min

Temperature (°C)	**Content (mg/g dried sample)**			
	TPC	Hydroxytyrosol	Tyrosol	Oleuropein
140	5,9	0,67	0,21	0,02
170	8,9	1,76	0,51	0,01
200	20,9	-	-	-

As expected, as the extraction temperature increases, the amount of TPC increases, Table 3.4. But it is noted that TPC content obtained from extraction at 200 °C with sub-CW is about two times higher than the result obtained by the conventional, method hydro-alcoholic extraction. One possible explanation is that the phenolic compounds present in the sample are trapped within the matrix, which makes conventional extraction difficult. Another explanation, is the fact that with the sub-CW, lignocellulosic structure is hydrolyzed, which causes certain nonstructural phenolic compounds to become accessible. Moreover, it can also occur some degradation of lignin which is a component of lignocellulosic rich in polyphenols.

HPLC analysis was also performed in order to identifying and quantifying the phenolic compounds, present in the extracts collected during the assays. For both assays (140 and 170 °C) the same compounds were obtained and again the hydroxytyrosol is the most abundant compound. As in the characterization of FS sample, the TPC content obtained by the Folin method is much higher than the total obtained from the HPLC analysis.

Figure 3.4 shows that the extraction of phenolic compounds is directly related to temperature since as can be seen, the increase in TPC content closely follows the temperature increase, i.e., the linear temperature variation being accompanied by a linear change TPC. But can also be noted, that if the extraction process was prolonged beyond the 140 min at a constant temperature, the trend would be the gradual increase of extracted TPC concentration over time. So it can be concluded that besides the temperature TPC extracted content also depends on the extraction time.

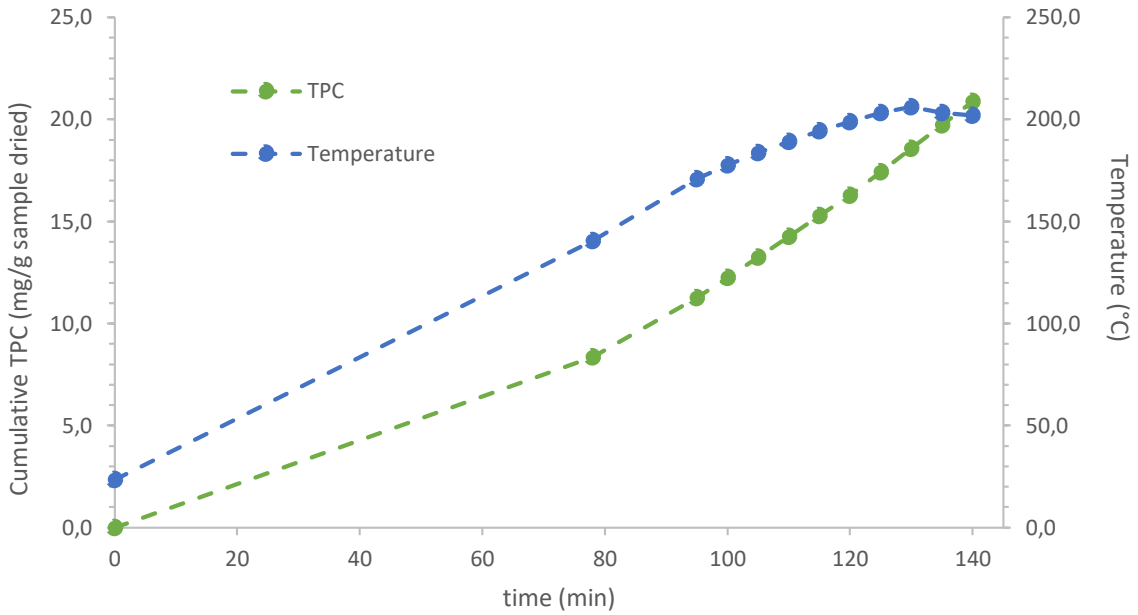

Figure 3.4 - Accumulation of phenolic compounds extracted per time for the assay at 200 °C. (P = 60 bar; Flow rate = 10 mL/min)

As expected, the extraction of phenolic compounds is favored in subcritical water conditions. And higher is the extraction temperature, higher is the concentration of phenolic compounds.

3.2.3 Lipid Content

In order to evaluate the potential of the residue remaining in the reactor after the extraction using sub-critical water, and checking the amount of oil that has been extracted during the process it was performed the quantification of lipids which remain in the residue. For this purpose, a *Soxhlet* extraction of the lipids was performed, following the procedure described in Chapter 2.2.1.

As mentioned in Chapter 1.7, with the increase in temperature, the dielectric constant of water decreases, which means that water behaves as a nonpolar solvent, i.e., the solubility of the ionic molecules decreases and consequently the solubility of hydrophobic molecules increases. Due to this fact, the sub-critical water is a good solvent to extract lipids.

Table 3.5 - Amount of oil (g oil/g initial dried biomass) in residue after the extraction with sub-CW and in initial biomass.

Temperature (°C)	In residue (wt. %)	In biomass (wt. %)
140	45.0%	55.2%
170	44.3%	53.9%
200	43.6%	53.7%

As can be seen on Table 3, the percentage oil that remains in the residue is lower relative to the percentage of oil obtained in composition analysis, about 55%, i.e., about 10% of the lipid content present in the sample was extracted during the process.

As can be seen in Figure 3.5, the obtained oil from the resultant residue from the extraction carried out at 200 °C has a brown color compared to oil obtained from residues of extractions 140 and 170 °C, which had a green color similar to oil color obtained from the Fine Sample, Figure 3.2.

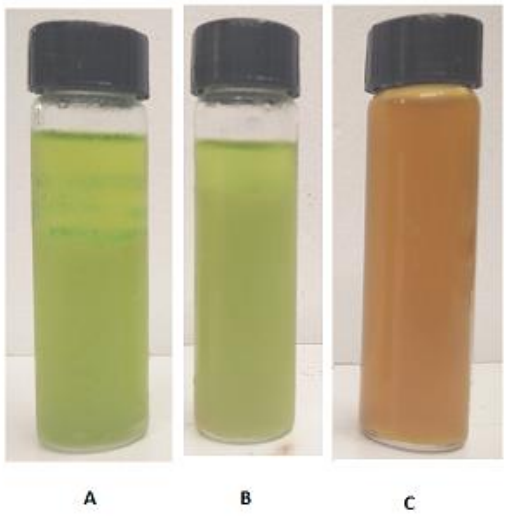

Figure 3.5 - Oil obtained from the residue of extractions. (P = 60 bar; Flow rate = 10 mL/min)
A - Residue from the assay at 140 °C; B - Residue from the assay 170 °C; C - Residue from the assay at 200 °C.

So it can be concluded that the residue resulting from the extraction using sub-critical water still shows great potential as lipid source. Which could possibly be extracted with other green solvent, such as supercritical CO_2. But to do this extraction, it would be necessary drying the residue, and this raises a question, if it would be compensatory the energy costs inherent to dryness in relation to the price of oil and the quantity obtained. Order to obtain a response would be necessary to make an energy and costs balance. Prior to this extraction, it is essential to analyze the oil, making the quantification of fatty acid by the method of Lepage and Roy and the respective identification of such acids by gas chromatography

(GC). It would also be important to check the presence of other compounds such as phenolic compounds with antioxidant potential that has not been extracted with the sub-critical water.

3.2.4 Carbohydrates Content

As mentioned in Chapter 1.1.1, the cellulose is joined by β-bonds in a crystalline structure, which makes it more difficult to hydrolyze into glucose, compared to hemicellulose, which is an amorphous polymer that easily hydrolyze in its sugar components. [14]

The use of higher temperatures causes an increase in the capacity of water to solubilize analytes and hydrolyze several components of plant materials, a great amount of diverse compounds can be released during sub-CW extraction. At temperatures of 160 °C and higher, sub-CW is even able to solubilize hemicellulose and lignin. Moreover, during a water-based thermal process, part of the hemicellulose is hydrolyzed and forms acids. These acids are assumed to catalyze the hydrolysis of remaining hemicelluloses. [103], [104]

In Chapter 3.1, it was observed that about 18% of the weight of the Fine sample from *"alperujo"* were carbohydrates, of which 13%, approximately, were structural sugars which are present as part of the structure of cellulose and hemicellulose.

In order to evaluate the ability to extract the carbohydrate fraction by extraction/hydrolysis with sub-CW, from the fine sample of the *"alperujo"*, the quantification of carbohydrates recovered during the assays was performed, using the methodology described in Chapter 2.3.4. It was also carried out the identification and quantitation of monosaccharides by the HPLC method described in Chapter 2.3.6A.

Table 3.6 - Total sugars recovered (g/g extract) during the assay for different temperatures. P = 60 bar; Flow rate = 10 mL/min

Temperature (°C)	**Total sugars recovered (wt. %)**
140	13%
170	24%
200	14%

As can be seen in Table 3.6, when the temperature increases from 140 to 170 °C, the percentage of total sugars recovered in the extract during assay also increases, but at 200 °C the total sugars recovered

decrease, which it is not expected, because as the temperature increases, the reactivity of the water also increases, so the total sugars recovered should increase.

One possible reason, is that during the extraction at 200 °C may have occurred degradation of the sugars present in biomass, this can be confirmed by the change of the oil color obtained from the residue of this assay after extraction by *Soxhlet* method, Figure 3.5.

But in terms of the initial amount of biomass, the best yield of total carbohydrates was achieved at 200 °C (5%), around 4% higher than at 140 °C.

3.2.5 Antioxidant Activity

The analysis of the antioxidant activity of phenolic compounds extracted through sub-CW, was also one of the objectives of this work. To do this analysis, the DPPH (2,2-diphenyl-1-picrylhydrazyl) assay was performed, following the methodology described in Chapter 2.3.7. To evaluate the antioxidant activity of the extracts obtained in each assay, the EC_{50} was calculated (extract concentration, mg/L, required to reduce the initial concentration of DPPH radical by 50%) from the inhibition curves obtained. A lower EC_{50} value corresponds to a higher activity, to eliminate the DPPH radical and therefore corresponds to good antioxidant activity. [102]

As mentioned in Chapter 1.6, the phenolic compounds are natural sources of antioxidants. Due to the antioxidant activity, phenolic compounds are able to eliminate free radicals, donate hydrogen atoms or electrons and chelate metal cations. Their structure are an important factor for these properties. Antioxidant activity is related to the different phenolic compounds, which are present in each extract, and varies with the substitutions that may occur on the aromatic ring as well as the type of lateral structure. [105], [106]

The extracts selected for the study, resulted from the lyophilization of the accumulation of liquors collected in the assay at 200 °C, according to the range temperature from Table 3.7. The total liquors obtained were also analyzed in order to quantify the TPC.

In the study by Vergara-Salinas *et al.* (2012), the temperature was found to be the dominant factor in attaining high antioxidant activity, wherein the antioxidant activity values increased (EC_{50} decreased) with increasing extraction temperature, because several reactions may generate compounds with antioxidant activities at temperatures above 100 °C, as can be observed in Table 3.7, for Total 1, 2 and 3,

that as the temperature increases, the EC_{50} value decreases, which is directly related to increasing the value of TPC. [103]

Table 3.7 - Totals liquors obtained from the accumulation of the liquors collected in the assay at 200°C. P = 60 bar; Flow rate = 10 mL/min

Name sample	Range temperature (°C)	TPC (mg/g dried biomass)	EC_{50} (mg extract/L)
Total 1	[0 – 140]	8.36	35.51
Total 2	[0 – 170]	13.33	34.54
Total 3	[0 – 200]	25.61	32.83
Total 4	[140 – 170]	2.91	28.79
Total 5	[170 – 200]	6.61	41.56

Comparing the results of TPC and EC_{50} for Total 4 and 5, it is observed that with an increase in temperature results in an increase in TPC content, as expected. However, the EC_{50} value also increases, i.e., the antioxidant activity decreases, which may be explained by acceleration of initiation reactions associated with faster utilization of antioxidants. The decrease in antioxidant activity with increasing temperature (which is faster for antioxidants with higher oxidation potential, i.e. antioxidants with lower reactivity against free radicals) is caused by a decrease in the ability of antioxidants to react with free radicals (particularly with peroxyl radicals of fatty acids) at higher temperatures. [107]

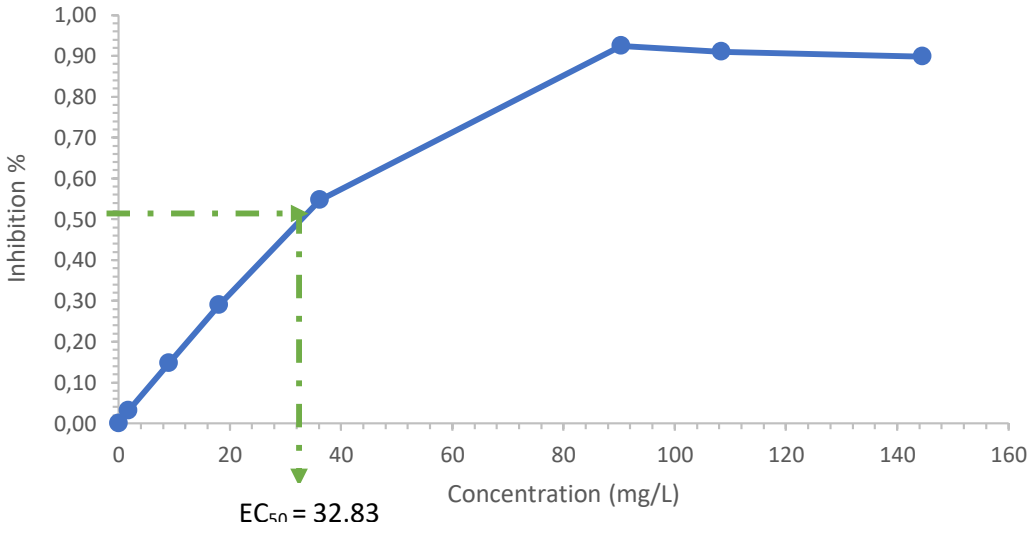

Figure 3.6 - Antioxidant activity of the extract obtained from Total 3, [0 - 200] °C.

It can then be concluded from Table 3.7, that the maximum antioxidant activity for Fine Sample from "*alperujo*" was obtained with extracts collected at 200 °C, which are the extracts with higher content of phenolics compounds.

3.3 Stability Assay

In the stability assay, the quantification of the total phenolic compounds by the Folin-Ciocalteu method was performed, following the protocol described in Chapter 2.3.5. In order to see the evolution of TPC over time, sample storage conditions were taken into account. This assay also studied the evolution of the antioxidant activity of different samples, following the protocol described in Chapter 2.3.7.

As mentioned above, the phenolic compounds are sensitive to temperature and light exposure, and, as can be seen by the Figure 3.7, the TPC value for the CT (sample stored at Cold Temperature) and RT (sample stored at Room Temperature) samples, decrease over time as was expected. Since the RT sample was stored at a higher temperature and was always been exposed to light, a more pronounced decrease in TPC value after 30 days should be observed when compared to the sample CT, this can be explained by the rapid degradation of phenolic compounds present in sample.

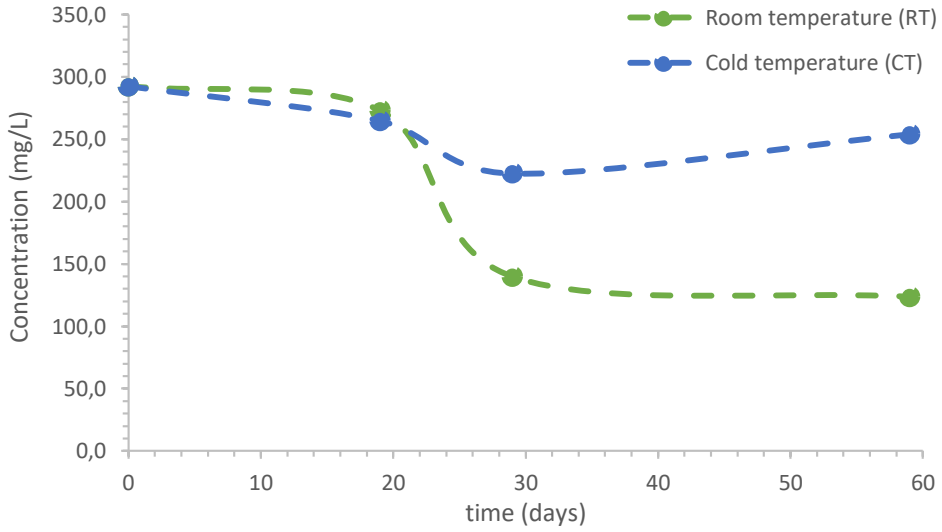

Figure 3.7 - TPC content per time.

In Chapter 3.2.2, it has been verified that the antioxidant activity may be positively correlated to the amount of total phenolic compounds

Table 3.8 - TPC and EC_{50} values per time for RT and CT samples.

time (days)	RT Sample		CT Sample	
	TPC (mg/L)	**EC_{50} (mg/L)**	**TPC (mg/L)**	**EC_{50} (mg/L)**
0	292.6	56.66	292.6	56.66
20	273.1	91.57	264.8	42.82
30	139.8	113.53	222.6	48.75
60	123.6	153.56	254.1	48.05

And as can be seen in Table 3.8, over time, as the EC_{50} value for the RT sample increases the TPC value decreases. Furthermore, the EC_{50} value for CT sample decreases with decreasing content of phenolic compounds, one possible cause is the proliferation of fungi on the sample, changing its composition and yielding a false-positive value.

Then, it was made a study of antioxidant activity by the DPPH assay, as well as, the content of total phenolic compounds by the Folin method, to different storage methods, the samples used for the study are:

- CT sample (sample stored at **C**old **T**emperatures) was stored for 68 days at 4 °C in a Schott flask covered in aluminum foil to protected the sample from light;
- RT sample (sample stored at **R**oom **T**emperatures) was subjected to nitrogen and stored for 68 days in a falcon tube at room temperature and unprotected from light;
- FRT sample (sample stored in the **F**reezer and at **R**oom **T**emperature) was store in a falcon tube at freezer temperature and protected from light, -20 °C, for 48 days and 20 days at room temperature and unprotected from light;
- FT sample (sample stored in the **F**reeze **T**emperature) was store in a falcon tube 68 days at freezer temperature and protected from light.

After extraction, the analysis of the TPC by the Folin method, described in Chapter 2.3.5 and antioxidant activity by the DPPH assay described in Chapter 2.3.7. The results obtained described a TPC value of 293 mg/L and an EC_{50} value of 57 mg/L. Both values are used as reference value (Zero sample).

Table 3.9 - TPC and EC_{50} taking into account the type of storage

Sample	TPC (mg/L)	EC_{50} (mg/L)
Zero	292.58	56.66
FRT	241.90	35.62
FT	230.59	36.15
RT	123.58	153.56
CT	254.05	48.05

Zero – Reference sample;
FRT – Sample storage at freezer temperature (-20 °C) for 48 days and room temperature for 20 days;
FT – Sample storage at freezer temperature (-20°C) for 68 days;
RT – Sample storage at room temperature for 68 days and unprotected from light;
CT – Sample storage at cold temperature (4°C) for 68 days.

In the Table 3.9, it can be observed that the samples stored at temperatures below room temperature (FRT, FT and CT samples) do not exhibit significant change in the TPC 68 days after the extraction, compared to the value obtained in the reference sample (Zero). There is also no major change in the EC_{50} values obtained for samples FRT, TF and TC, compared to the EC_{50} for the reference sample. As expected the sample RT presents a very high EC_{50} value compared to the others samples, which is directly related to the decrease in the value of TPC that can be seen in Table 3.9.

It is concluded then that phenolic compounds suffer less degradation when the samples are stored at temperatures below room temperature and protected from light.

4 CONCLUSIONS AND FUTURE WORK

The "*alperujo*" is a byproduct of olive oil extraction, more specifically, of 2-phase extraction process, which is described as being a clean and green process, because eliminates liquid effluent produced (wastewater) which are highly pollutant. This by-product is characterized by being a slurry with high water content and is a mixture of skin, flesh and seeds from the olives. It consists of residual oil and phenolic compounds with antioxidant capacity. Because of these polyphenols, the "*alperujo*" has great value for the cosmetic, food and pharmaceutical industries. For this reason, the processing of this biomass is promising.

One method for the recovery of compounds with added value present in "*alperujo*", such as the phenolics compounds, is the extraction using the subcritical water.

In this work, it can be concluded that the phenolic compounds extraction with subcritical water is shown to be efficient. About 8.36 mg/g of phenolic compounds were extracted at 140 °C, which corresponds to 77% of the total phenolic compounds obtained in the characterization of Fine Sample, furthermore at this temperature the EC_{50} value was of 35.51 mg/L. However, at 200 °C, 25.61 mg/g of TPC was obtained and the EC_{50} value of 32.83 mg/L.

The EC_{50} value is the antioxidant quantities required to reduce 50% of the initial concentration DPPH, the differences between the EC_{50} obtained for 140 °C is 2.68 mg/L compared to the value obtained for 200 °C. Both the extractions at 140 °C and 200 °C were effective, but to know which of the two temperatures is more advantageous, it would be necessary to evaluate the energy costs of heating through energy balances, and the price of the product.

The extraction carried out at 200 °C showed higher conversion, 43%, and a yield of soluble compounds of 30%, which was due to the heating time and the duration of the assay, as the higher is the target temperature, the greater is the assay duration, and in turn lead to a larger amount extracted. On the other hand, the assay at 140 °C shows a biomass conversion of 19% and a yield of water-soluble compounds of 5%. Both values are lower compared to the data obtained to 200 °C.

It has also been proved the existence of a relationship between the increased of the temperature extraction with increasing TPC content, which in turn is associated with antioxidant activity increased, i.e., decreased EC_{50} value. It was found that 68 days after the extraction the phenolic compounds degrade less, and in turn their antioxidant activity decreases slightly, when the samples collected during the assay are stored at temperatures below room temperature, preferably -20 °C and protected from exposure to light.

It was also observed that the residue obtained from the hydrolysis has a great potential as a source of lipid, that can be extracted with another green solvent, for example supercritical CO_2. The Fine Sample contained on average 54% of the mass content of lipids of which 10% are extracted for the three temperatures studied.

In the future, it is important to make a study of the Gross Sample (GS), which is constituted majorly by olive pits, with aim to determine which of the alperujo constituent (pits or flesh) is more promising for the extraction of antioxidants. It would also fundamentally optimize the process for both samples. It would also be relevant to study the constitution of the *"alperujo"* for different olives qualities, because the phenolic composition varies depending on the olive species, maturation time, climate, soil and other factors.

In the test of stability, it was found that over time the TPC value of the sample stored at cold temperature (CT sample) decreased, but the EC_{50} increased, indicating an increased antioxidant activity, so it would be necessary to make a broader stability study, wherein, it would be necessary to quantify the phenolic compounds over time, as well as the proliferation of fungus, which can affect the DPPH assay.

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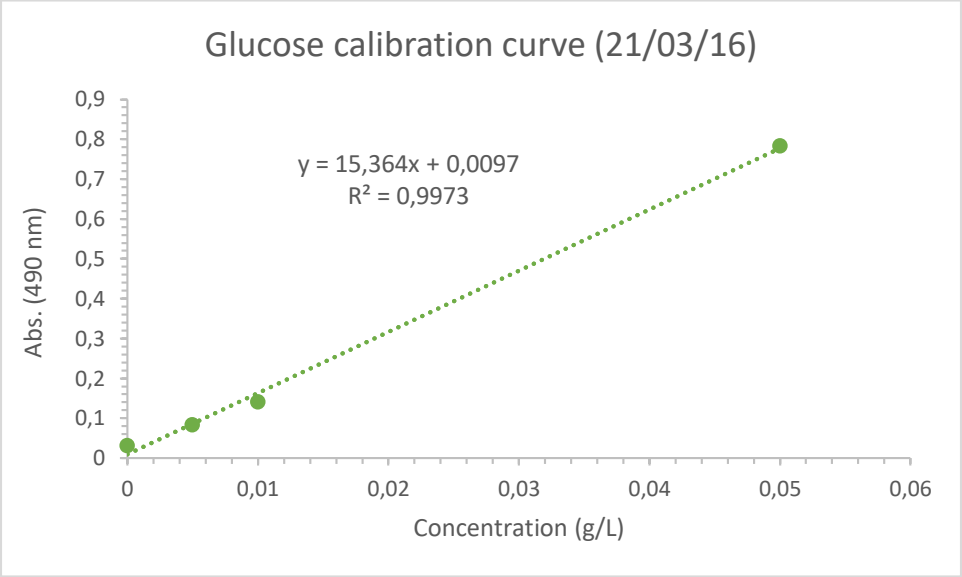
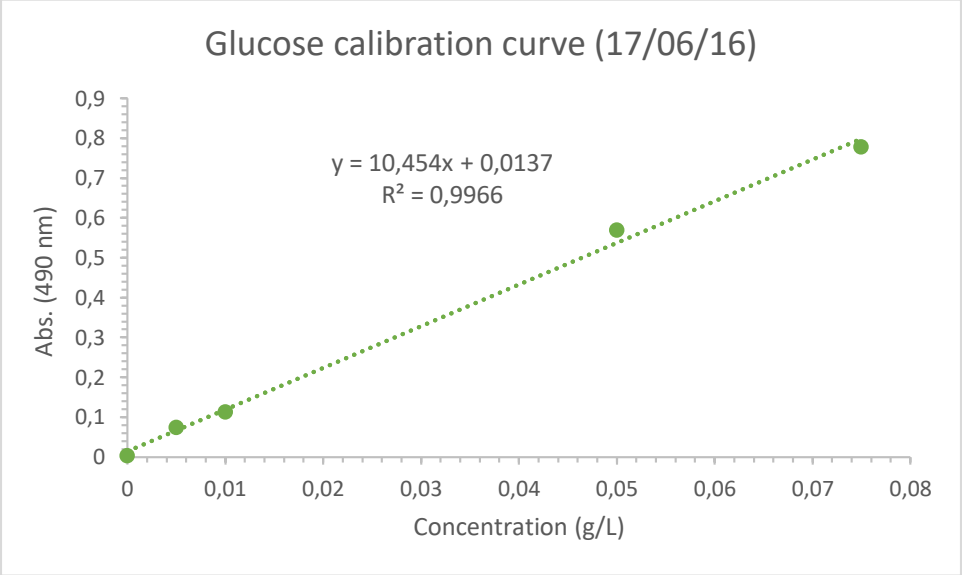
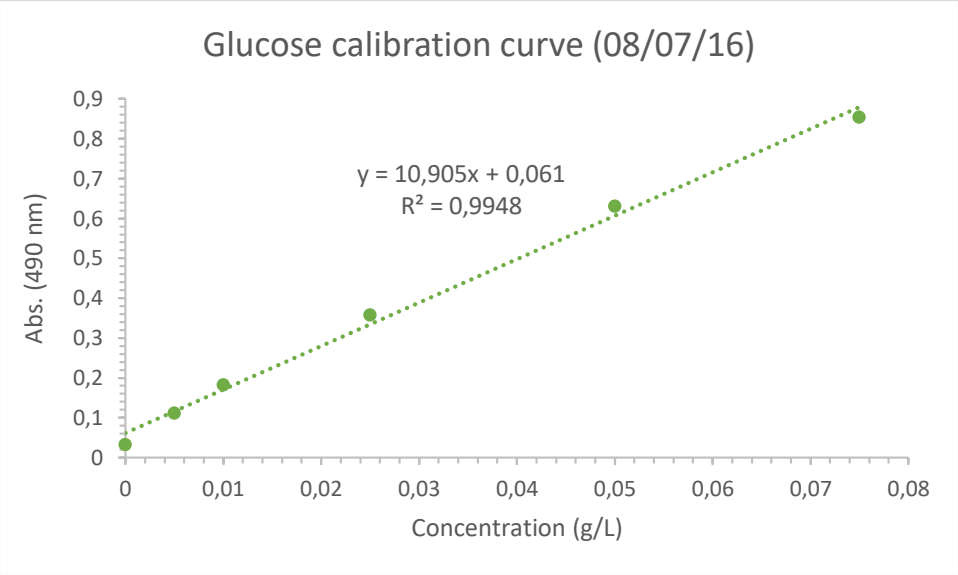
6 APPENDIX

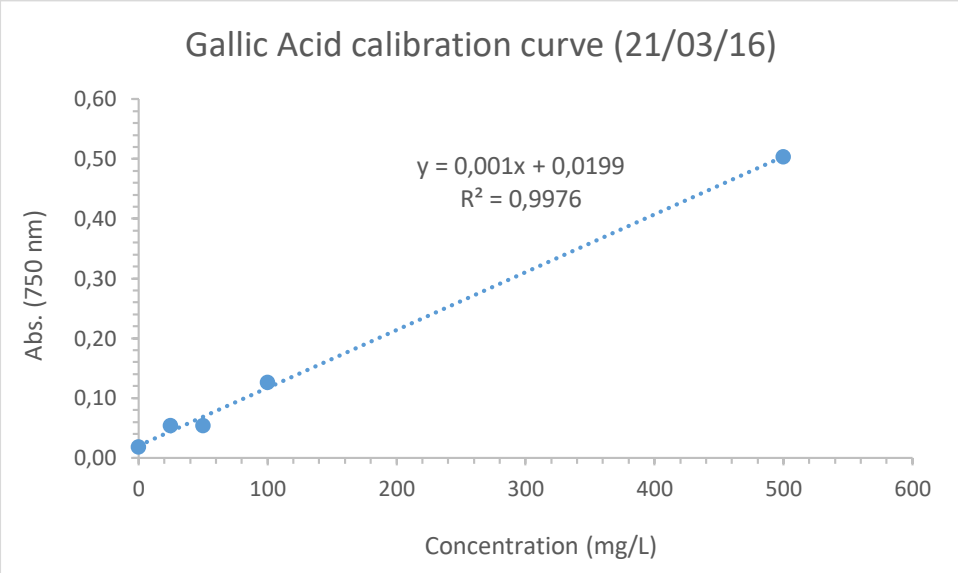
6.1 Calibration curves – Standard for phenol-sulphuric method

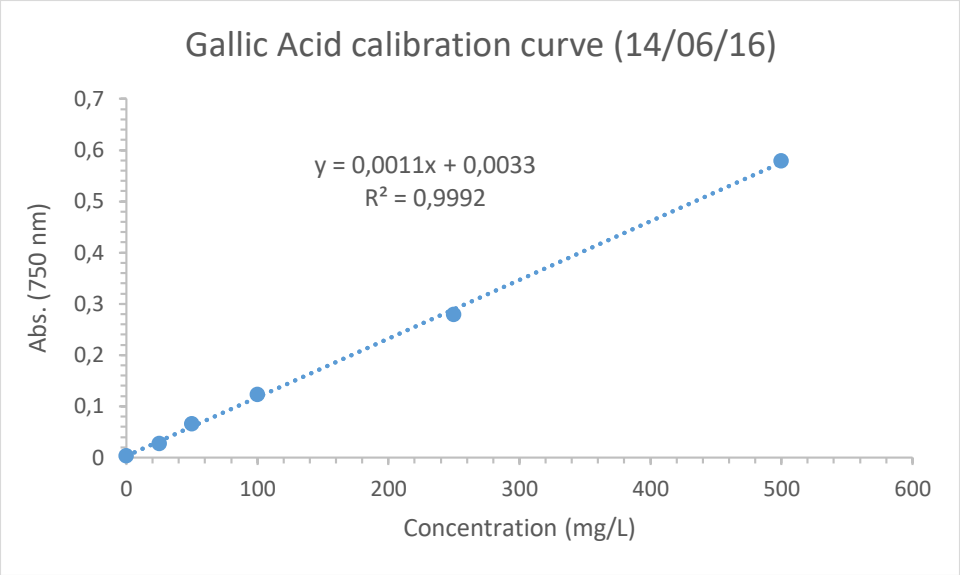


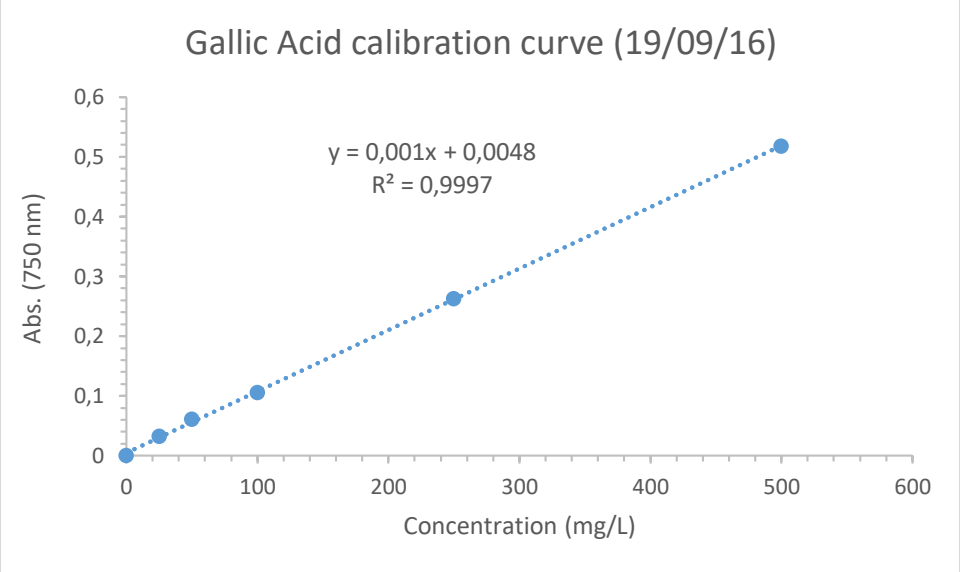




6.2 Calibration curves – Standard for the Folin-Ciocalteu method




Gallic Acid calibration curve (14/06/16)
y = 0,0011x + 0,0033
R² = 0,9992
Abs. (750 nm)
Concentration (mg/L)
0
0,1
0,2
0,3
0,4
0,5
0,6
0,7
0
100
200
300
400
500
600


Gallic Acid calibration curve (19/09/16)
y = 0,001x + 0,0048
R² = 0,9997
Abs. (750 nm)
Concentration (mg/L)
0
0,1
0,2
0,3
0,4
0,5
0,6
0
100
200
300
400
500
600

6.3 Composition of “alperujo” – HPLC Chromatograms results

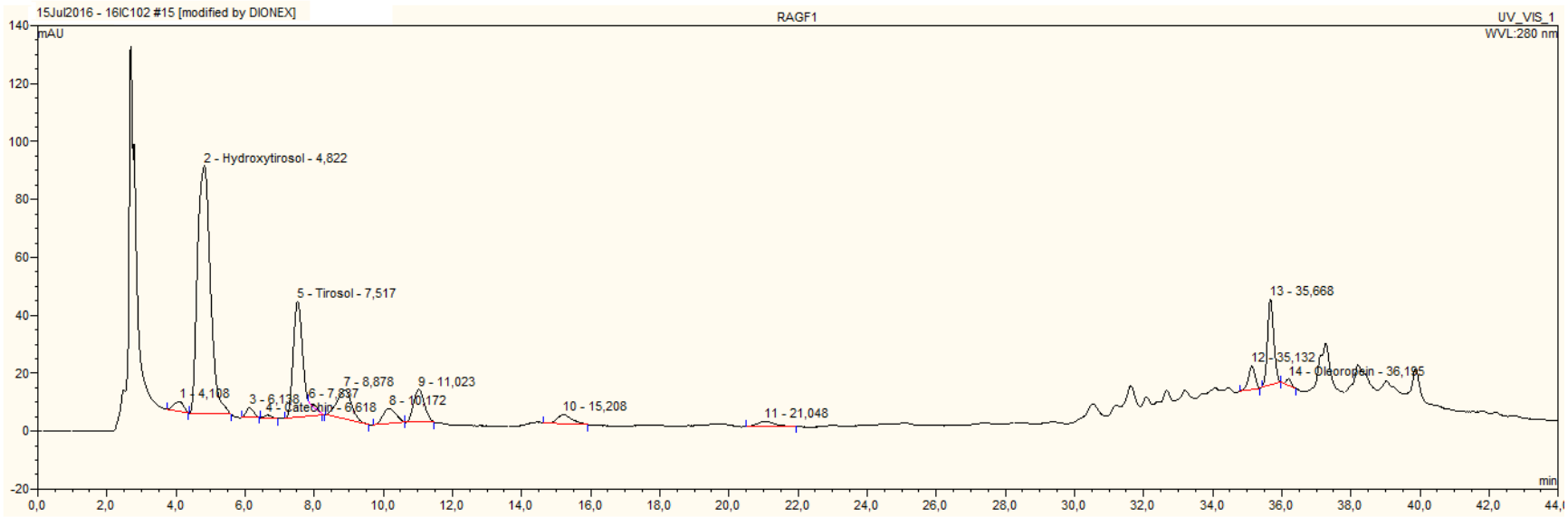


Figure 6.1 – HPLC chromatograms at 280 nm of Gross Sample (GS) of "alperujo".

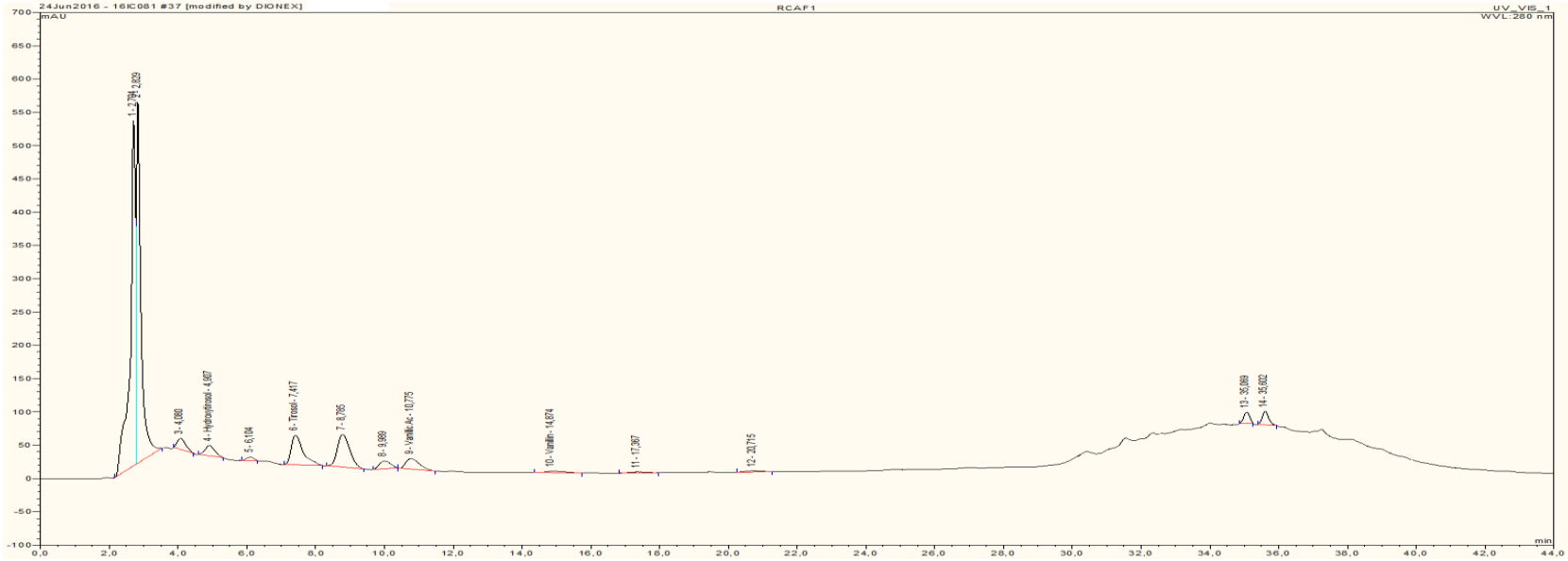


Figure 6.2 - HPLC chromatograms at 280 nm of Fine Sample (FS) of "alperujo".